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(54) Title: BLUE COPPER OXIDASE MUTANTS WITH ENHANCED ACTIVITY

## (57) Abstract

The present invention relates to mutants of a blue multi-copper oxidase, comprising (a) a substitution of one or more amino acid residues with other amino acid residues, (b) and insertion of one or more amino acid residues and/or (c) a deletion of one or more amino acid residues, wherein the substitution, insertion or deletion is carried out at a position which is located no greater than 15Å from a Type I (T1) copper site. The present invention also relates to nucleic acid constructs comprising a nucleic acid sequence encoding the mutants of the present invention, host cells comprising the construct of the present invention, and methods for producing mutants of the present invention.



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## BLUE COPPER OXIDASE MUTANTS WITH ENHANCED ACTIVITY

### Background of the Invention

#### 5 Field of the Invention

The present invention relates to mutant multi-copper oxidases. More specifically, the invention relates to oxidases which have been modified so as to exhibit altered pH activity profiles relative to the wild-type oxidase.

#### 10 Description of the Related Art

There are currently a number of well-known blue copper oxidases which have various commercial/industrial applications. Two major classes of these enzymes are recognized: (1) the single copper proteins, which are single copper-containing, blue electron-transfer proteins such as plastocyanin, azurin, stellacyanin, amicyanin, auracyanin, cucumber basic blue, mavicyanin, rusticyanin, and umecyanin; and (2) the multi-copper oxidases, which are multiple copper-containing, blue oxidoreductases such as laccase, bilirubin oxidase, phenoxazinone synthase, ascorbate oxidase, ceruloplasmin, and nitrite reductase. The blue color of these proteins arises from the so-called Type 1 (T1) copper site.

It is an object of the present invention to provide mutants of blue multi-copper oxidases which have improved properties.

### Brief Description of the Figures

Figure 1 shows the scheme for construction of intermediate plasmid pInt2.22 and oligonucleotide-directed mutagenesis of the *Myceliophthora thermophila lcc-1* gene.

Figure 2 shows the construction of the intermediate pInt1 which contains the *Aspergillus oryzae* TAKA amylase promoter and 5'-portion of the *Myceliophthora thermophila lcc-1* coding region.

Figure 3 shows the final step in construction of pRaMB17 and its derivatives, pRaMB17M and pRaMB17Q, which direct expression of wild-type and mutant forms of *Myceliophthora thermophila* laccase (MtL).

Figure 4 shows the construction of pBANe22T which directs expression of a mutant form of *Myceliophthora thermophila* laccase.

Figure 5 shows the pH activity profiles of the wild-type (wt) and mutant *Rhizoctonia solani* laccases (RsLs) and *Myceliophthora thermophila* laccases (MtLs): wt (—); mutant M (—); mutant T (.....); (A), RsL with 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS); (B), RsLs with syringaldazine (SGZ); (C), MtLs with ABTS; (D), MtLs with SGZ.

Figure 6 shows the nucleotide sequence and the deduced amino acid sequence of *Rhizoctonia solani* laccase isozyme 4 (*rsl4*) gene (SEQ ID NOS:24 and 25).

Figure 7 shows the nucleotide sequence and the deduced amino acid sequence of *Myceliophthora thermophila* laccase *lcc-1* gene (SEQ ID NOS:26 and 27).

### Summary of the Invention

The present invention relates to mutants of a blue multi-copper oxidase, comprising a mutation selected from the group consisting of (a) a substitution of one or more amino acid residues with other amino acid residues, (b) an insertion of one or more amino acid residues and/or (c) a deletion of one or more amino acid residues, wherein the substitution, insertion or deletion is carried out at a position which is located no greater than 15Å from a Type I (T1) copper site. The present invention also relates to nucleic acid constructs comprising a nucleic acid sequence encoding the mutants of the present invention, host cells comprising the construct of the present invention, and methods for producing mutants of the present invention.

### Detailed Description of the Invention

The present invention relates to mutants of a blue multi-copper oxidase, comprising (a) a substitution of one or more amino acid residues with other amino acid residues, (b) an insertion of one or more amino acid residues and/or (c) a deletion of one or more amino acid residues, wherein the substitution, insertion or deletion is carried out at a position which is located no greater than 20Å from a Type I (T1) copper site. Preferably, each mutation is a substitution of one or more amino acid residues with other amino acid residues.

The Type 1 copper site consists of four ligands which bind to a copper ion, each of which is either an amino acid residue of the blue copper oxidase or a small molecule such

as a water molecule. A ligand is defined herein an amino acid residue of a blue copper oxidase which binds to a copper ion. The Type 1 copper site of all known blue copper oxidases consists of the following ligands: two histidines (H), one cysteine (C), and, possibly, one additional methionine.

5 The ligand location for *Rhizoctonia solani* is: H 427, C 480, H 485 and possibly L 470 and for *Myceliophthora thermophilum* is H 431, C 503, H 508 and possibly L 513.

For purposes of the present invention, the distance from a Type I copper site is measured from the copper ion.

10 In a preferred embodiment, the mutant has a mutation at a position which is located no greater than 15Å from a Type I copper site. In another preferred embodiment, the mutant has a mutation at a position which is located no greater than 12Å from a Type I copper site. In another preferred embodiment, the mutant has a mutation at a position which is located no greater than 10Å from a Type I copper site. In another preferred embodiment, the mutant has a mutation at a position which is located no greater than 8Å from a Type I copper site.  
15 In another preferred embodiment, the mutant has a mutation at a position which is located no greater than 6Å from a Type I copper site. In another preferred embodiment, the mutant has a mutation at a position which is located no greater than 4Å from a Type I copper site. In another preferred embodiment, the mutant has a mutation at a position which is located no greater than 2.5Å from a Type I copper site. In another preferred embodiment, the mutant has a mutation of an amino acid residue which is adjacent to a Type I copper site ligand. In another preferred embodiment, the mutant has a mutation of an amino acid residue which is a Type I copper site ligand.

20 The mutants of the present invention are mutants of a blue multi-copper oxidase. Preferably, the blue multi-copper oxidase is a bilirubin oxidase (Kokeida *et al.*, 1993, 25 *Journal of Biological Chemistry* 268: 18801-18809). In another preferred embodiment, the blue multi-copper oxidase is a phenoxazinone synthase (Freeman *et al.*, 1993, *Biochemistry* 32: 4826-4830). In another preferred embodiment, the blue multi-copper oxidase is an ascorbate oxidase (Tauber *et al.*, 1935, *Journal of Biological Chemistry* 110: 211). In another preferred embodiment, the blue multi-copper oxidase is a ceruloplasmin (Curzon and Young, 1972, *Biochimica Biophysica Acta* 268: 41). In another preferred embodiment, the blue multi-copper oxidase is a nitrite reductase (Godden *et al.*, 1991, *Science* 253: 438-442).  
30 In another preferred embodiment, the blue multi-copper oxidase is a laccase. In a most

preferred embodiment, the blue multi-copper oxidase is a fungal laccase, e.g., a *Rhizoctonia* laccase (preferably a *Rhizoctonia solani* laccase or RsL; WO 95/07988) or a *Myceliophthora* laccase (preferably a *Myceliophthora thermophilum* laccase or MtL described in U.S. application Serial No. 08/253,781, which is incorporated herein by reference).

5 In another preferred embodiment, the oxidase is another *Rhizoctonia* laccase (as disclosed in U.S. application Serial No. 08/172,331, which is incorporated herein by reference), another *Myceliophthora* laccase (as disclosed in U.S. application Serial No. 08/253,781, which is incorporated herein by reference), and laccases of *Polyporus* (as disclosed in U.S. application Serial No. 08/441,147, which is incorporated herein by reference), *Trametes*, *Pyricularia*, *Coriolus*, *Scytalidium* (as disclosed in U.S. application Serial No. 08/253,784, which is incorporated herein by reference), *Rigidoporus* and *Phenllinus* (Geiger et al., 1986, *Appl. Biochem. Biotech.*, 13: 97-110), *Podospora* (Moltitoris and Reinhammar, 1974, *Biochimica Biophysica Acta* 386: 493-502), *Lentinus* (Leatham and Stahmann, 1980, *Journal of General Microbiology* 125: 147-157), *Neurospora* (Germann et al., 1987, *Journal of Biological Chemistry* 263: 885-896), *Aspergillus* (Kurtz and Champe, 1982, *Journal of Bacteriology* 151: 1338-1345), *Phlebia* (Niku-Paavola et al., 1988, *Biochemical Journal* 254:877-884), *Botrytis* (Dubernet et al., 1976, *Phytochemistry* 16: 191-193,), *Sclerotia* (Chet and Huttermann, 1982, *FEMS Microbiological Letters* 14: 211-215), *Curvularia* (Banerjee and Vohra, 1991, *Folia Microbiol.* 36: 343-346), *Fomes* (Haars and Huttermann, 1983, *Arch. Microbiol.* 134: 309-313), *Schizophyllum* (De Vries et al., 1986, *Journal of General Microbiology* 132: 2817-2826), *Cerrena* (Bekker et al., 1990, *Biokhimia* 55: 2019-2024), *Armillaria* (Rehman and Thurston, 1992, *Journal of General Microbiology* 138: 1251-1257), *Agaricus* (Perry et al., 1993, *Journal of General Microbiology* 139: 1209-1218), *Pleurotus* (Von Hunolstein et al., 1986, *Journal of General Applied Microbiology* 32: 185-191), *Acer pseudoplatanus* (Lafayette et al., 1995, *Plant Physiology* (Rockville) 107: 667-668), and *Rhus* (Bertrand, 1895, *C. R. Acad. Sci. Paris* 121: 166).

The mutants of the present invention may have a different specific activity than the wild-type blue copper oxidases. For example, a negative charge, or more precisely, a relatively high electron density, in the T1 copper site region is important for activity.

30 Furthermore, the mutants of the present invention may have a different pH-activity profile than the wild-type blue copper oxidases, e.g., the mutants can have a higher or lower pH optimum by an alteration of the charge distribution (or dielectric anisotropy) at the T1

copper site. In order to enhance the activity of the oxidase of interest in a more alkaline pH range, electron density and/or negative charge should be increased. Thus, in the mutants of the present invention, (a) a neutral amino acid residue is substituted with a negative amino acid residue or (b) a positive amino acid residue is substituted with a negative or neutral amino acid residue. In addition, neutral residues equipped with a functional group that bear a relatively high electron density and could act as general base, such as histidine, serine, threonine, tyrosine, cysteine, and methionine, may also be used to substitute other neutral residues possessing only simple aliphatic or aromatic side chains, such as leucine and phenylalanine. In order to enhance the activity of the oxidase of interest in a more acidic pH range, electron density and/or negative charge should be decreased. Thus, in this embodiment of the mutants of the present invention, (a) a neutral amino acid residue is substituted with a positive amino acid residue or (b) a negative amino acid residue is substituted with a positive or neutral amino acid residue.

The present invention also relates to mutants which can be expressed in higher yields. Such mutants include oxidases comprising a substitution of a phenylalanine with another amino acid residue. For example, substituting phenylalanine at a position corresponding to residue 513 of *Myceliophthora thermophila* laccase and position 470 in *Rhizoctonia solani* isozyme 4 laccase results in a low expression yield. Thus, the mutants of the present invention encompass substitutions of Phe at one of these positions with another amino acid residue. Preferably, the amino acid residue does not ligate to copper, i.e., the amino acid residue is not histidine, cysteine, methionine, glutamate, and aspartate. Preferably, phenylalanine is substituted by leucine. In a preferred embodiment, the yield of the mutant enzyme is increased at least two-fold, more preferably at least five-fold, over the yield observed with the corresponding wild-type enzyme when both are expressed in the same host and fermented under the same conditions.

In a preferred embodiment, the mutants of the present invention comprise a mutation in a region corresponding to: (a) the segment that contains one Cu-ligating His, e.g., 416VIELNITGGADHPI429 of *Rhizoctonia solani* laccase and 421ENDPGAPFTLPHPM433 of *Myceliophthora thermophila* laccase; (b) the segment that contains another ligating His and the ligating Cys, e.g., 474GPWFVHCHIDWHLEAGLALVF494 of *Rhizoctonia solani* laccase and 497GAWLFHCHIAWHVSGGLGV515 of *Myceliophthora thermophila* laccase; (c) the segment corresponding to the sequence where Q353 and W362 of ascorbate oxidase

reside, e.g., 356VSLNLAIGRSTVDGIL371 of *Rhizoctonia solani* laccase and 361VTLDTTGTPLFWKVN376 of *Myceliophthora thermophila* laccase; (d) the segment corresponding to the sequence where R285 of ascorbate oxidase resides, e.g., 303LDPTNVFAVL312 of *Rhizoctonia solani* laccase and 308AIFHYAGAPG317 of *Myceliophthora thermophila* laccase; (e) the segment corresponding to the sequence where W163 of ascorbate oxidase resides, e.g., 217INVKRGKRYR226 of *Rhizoctonia solani* laccase and 222GRRHRLRLIN231 of *Myceliophthora thermophila* laccase; and (f) the segment corresponding to 465LEAGL472, more preferably 466LEAGL470, of *Rhizoctonia solani* laccase. Those skilled in the art will readily recognize, by routine homology alignment, the corresponding regions in other blue copper oxidases. In a preferred embodiment, the mutants comprise a mutation in the segment corresponding to 416VIELNITGGADHPI429 of *Rhizoctonia solani* laccase and 421ENDPGAPFTLPHM433 of *Myceliophthora thermophila* laccase.

In a preferred embodiment, the mutants comprise at least two amino acid residues, more preferably at least 3 amino acid residues. In another preferred embodiment, the mutants comprise five mutations, more preferably four mutations, even more preferably three mutations, even more preferably two mutations, and most preferably one mutation.

The mutants described herein are most efficiently prepared by site-directed mutagenesis of the DNA encoding the wild-type laccase of interest. Such techniques are well-known in the art, and are described in, for example, Sambrook *et al.*, 1989, *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York. The present invention also encompasses the nucleic acid encoding the mutant laccases, as well as vectors and host cells comprising same, for use in recombinant expression of the mutant enzyme.

The choice of host cells and expression vectors will to a large extent depend upon the enzyme of choice and its source. The mutant gene can be expressed, in active form, using an expression vector. A useful expression vector contains an element that permits stable integration of the vector into the host cell genome or autonomous replication of the vector in a host cell independent of the genome of the host cell, and preferably one or more phenotypic markers which permit easy selection of transformed host cells. The expression vector may also include control sequences encoding a promoter, ribosome binding site, translation initiation signal, and, optionally, a repressor gene, a selectable marker or various

activator genes. To permit the secretion of the expressed protein, nucleotides encoding a signal sequence may be inserted prior to the coding sequence of the gene. For expression under the direction of control sequences, a laccase gene to be used according to the invention is operably linked to the control sequences in the proper reading frame. Promoter sequences 5 that can be incorporated into plasmid vectors, and which can direct the transcription of the laccase gene, include, but are not limited to, the prokaryotic  $\beta$ -lactamase promoter (Villa-Kamaroff *et al.*, 1978, *Proceedings of the National Academy of Sciences USA* 75: 3727-3731) and the *tac* promoter (DeBoer *et al.*, 1983, *Proceedings of the National Academy of Sciences USA* 80: 21-25). Further references can also be found in "Useful proteins from recombinant 10 bacteria" in 1980, *Scientific American* 242: 74-94; and in Sambrook *et al.*, 1989, *supra*.

The expression vector carrying the nucleic acid construct of the invention may be any vector which may be conveniently subjected to recombinant DNA procedures. The vector may be an autonomously replicating vector, *i.e.*, a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, 15 *e.g.*, a plasmid, or an extrachromosomal element, minichromosome or an artificial chromosome. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

In the vector, the nucleic acid sequence should be operably connected to a suitable 20 promoter sequence. The promoter may be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell. Examples of suitable promoters for directing the transcription of the DNA construct of the invention, especially in a bacterial host, are the promoter of the lac operon of *E. coli*, the *Streptomyces coelicolor* agarase gene *dagA* 25 promoters, the promoters of the *Bacillus licheniformis* alpha-amylase gene (*amyL*), the promoters of the *Bacillus stearothermophilus* maltogenic amylase gene (*amyM*), the promoters of the *Bacillus amyloliquefaciens* alpha-amylase (*amyQ*), or the promoters of the *Bacillus subtilis* *xylA* and *xylB* genes. In a yeast host, a useful promoter is the ENO-1 promoter. For transcription in a fungal host, examples of useful promoters are those derived from the gene 30 encoding *Aspergillus oryzae* TAKA amylase, *Rhizomucor miehei* aspartic proteinase, *Aspergillus niger* neutral alpha-amylase, *Aspergillus niger* acid stable alpha-amylase, *Aspergillus niger* or *Aspergillus awamori* glucoamylase (*glaA*), *Rhizomucor miehei* lipase,

*Aspergillus oryzae* alkaline protease, *Aspergillus oryzae* triose phosphate isomerase or *Aspergillus nidulans* acetamidase. Preferred are the TAKA-amylase and *glaA* promoters.

The expression vector of the invention may also comprise a suitable transcription terminator and, in eukaryotes, polyadenylation sequences operably connected to the DNA sequence encoding the laccase of the invention. Termination and polyadenylation sequences may suitably be derived from the same sources as the promoter. The vector may further comprise a DNA sequence enabling the vector to replicate in the host cell in question. Examples of such sequences are the origins of replication of plasmids pUC19, pACYC177, pUB110, pE194, pAMB1 and pIJ702.

The vector may also comprise a selectable marker, *e.g.*, a gene the product of which complements a defect in the host cell, such as the *dal* genes from *Bacillus subtilis* or *Bacillus licheniformis*, or one which confers antibiotic resistance such as ampicillin, kanamycin, chloramphenicol or tetracycline resistance. Examples of *Aspergillus* selection markers include *amdS*, *pyrG*, *argB*, *niaD*, *sC*, and *hygB* a marker giving rise to hygromycin resistance. Preferred for use in an *Aspergillus* host cell are the *amdS* and *pyrG* markers of *Aspergillus nidulans* or *Aspergillus oryzae*. A frequently used mammalian marker is the dihydrofolate reductase (DHFR) gene. Furthermore, selection may be accomplished by co-transformation, *e.g.*, as described in WO 91/17243.

It is generally preferred that expression gives rise to a product that is extracellular. The laccases of the present invention may thus comprise a preregion permitting secretion of the expressed protein into the culture medium. If desirable, this preregion may be native to the laccase of the invention or substituted with a different preregion or signal sequence, conveniently accomplished by substitution of the DNA sequences encoding the respective preregions. For example, the preregion may be derived from a glucoamylase or an amylase gene from an *Aspergillus* species, an amylase gene from a *Bacillus* species, a lipase or proteinase gene from *Rhizomucor miehei*, the gene for the alpha-factor from *Saccharomyces cerevisiae*, or the calf prochymosin gene. Particularly preferred, when the host is a fungal cell, is the preregion for *Aspergillus oryzae* TAKA amylase, *Aspergillus niger* neutral amylase, the maltogenic amylase form *Bacillus NCIB 11837*, *Bacillus stearothermophilus* alpha-amylase, or *Bacillus licheniformis* subtilisin. An effective signal sequence is the *Aspergillus oryzae* TAKA amylase signal, the *Rhizomucor miehei* aspartic proteinase signal, and the *Rhizomucor miehei* lipase signal.

The procedures used to ligate the nucleic acid construct of the invention, the promoter, terminator and other elements, respectively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (cf., for instance, Sambrook *et al.*, 1989, *supra*).

5       The cell of the invention either comprising a nucleic acid construct or an expression vector of the invention as defined above is advantageously used as a host cell in the recombinant production of an enzyme of the invention. The cell may be transformed with the nucleic acid construct of the invention, conveniently by integrating the construct into the host chromosome. This integration is generally considered to be an advantage as the sequence is more likely to be stably maintained in the cell. Integration of the constructs into 10      the host chromosome occurs by homologous or heterologous recombination. Alternatively, the cell may be transformed with an expression vector as described above in connection with the different types of host cells.

15      The host cell may be selected from prokaryotic cells, such as bacterial cells. Examples of suitable bacteria are gram positive bacteria such as *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus lenthus*, *Bacillus brevis*, *Bacillus stearothermophilus*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus coagulans*, *Bacillus circulans*, *Bacillus laetus*, *Bacillus megaterium*, *Bacillus thuringiensis*, or *Streptomyces lividans* or *Streptomyces murinus*, or gram negative bacteria such as *E. coli*. The transformation of the bacteria may, 20      for instance, be effected by protoplast transformation or by using competent cells in a manner known *per se*.

25      The host cell is preferably a eukaryote, such as mammalian cells, insect cells, plant cells or preferably fungal cells, including yeast and filamentous fungi. For example, useful mammalian cells include CHO or COS cells. A yeast host cell may be selected from a species of *Saccharomyces* or *Schizosaccharomyces*, e.g., *Saccharomyces cerevisiae*. Useful filamentous fungi may be selected from a species of *Aspergillus*, e.g., *Aspergillus oryzae* or *Aspergillus niger*. Alternatively, a strain of a *Fusarium* species, e.g., *Fusarium oxysporum*, or *Fusarium graminearum*, can be used as a host cell. Fungal cells may be transformed by a process involving protoplast formation and transformation of the protoplasts followed by 30      regeneration of the cell wall in a manner known *per se*. A suitable procedure for transformation of *Aspergillus* host cells is described in EP 238 023. A suitable method of

transforming *Fusarium* species is described by Malardier *et al.*, 1989, *Gene* 78:147-156 or in copending U.S. application Serial No. 08/269,449.

The present invention thus also provides a method of producing a recombinant protein of the invention, which method comprises cultivating a host cell as described above under conditions conducive to the production of the enzyme and recovering the enzyme from the cells and/or culture medium. The medium used to cultivate the cells may be any conventional medium suitable for growing the host cell in question and obtaining expression of the laccase of the invention. Suitable media are available from commercial suppliers or may be prepared according to published formulae (*e.g.*, in catalogues of the American Type Culture Collection).

The resulting enzyme may be recovered from the medium by conventional procedures including separating the cells from the medium by centrifugation or filtration, precipitating the proteinaceous components of the supernatant or filtrate by means of a salt, *e.g.*, ammonium sulphate, followed by purification by a variety of chromatographic procedures, *e.g.*, ion exchange chromatography, gel filtration chromatography, affinity chromatography, or the like. Preferably, the isolated protein is about 90% pure as determined by SDS-PAGE, purity being most important in food, juice or detergent applications.

In a particularly preferred embodiment, the expression of the enzyme is achieved in a fungal host cell, such as *Aspergillus*. As described in detail in the following examples, the laccase gene is ligated into a plasmid containing the *Aspergillus oryzae* TAKA alpha-amylase promoter, and the *Aspergillus nidulans* *amdS* selectable marker. Alternatively, the *amdS* may be on a separate plasmid and used in co-transformation. The plasmid (or plasmids) is used to transform an *Aspergillus* species host cell, such as *Aspergillus oryzae* or *Aspergillus niger* in accordance with methods described in Yelton *et al.*, 1984, *Proceedings of the National Academy of Sciences USA* 81: 1470-1474.

The modified oxidases, particularly laccases of the present invention can be used in a number of industrial methods. These processes include polymerization of lignin, both Kraft and lignosulfates, in solution, in order to produce a lignin with a higher molecular weight. Such methods are described in, for example, Jin *et al.*, 1991, *Holzforschung* 45: 467-468; U.S. Patent No. 4,432,921; EP 0 275 544; PCT/DK93/00217, 1992.

The oxidases of the present invention can also be used for in situ depolymerization of lignin in Kraft pulp, thereby producing a pulp with lower lignin content. This use of these

enzymes is an improvement over the current use of chlorine for depolymerization of lignin, which leads to the production of chlorinated aromatic compounds, which are an environmentally undesirable by-product of paper mills. Such uses are described in, for example, *Current Opinion in Biotechnology* 3: 261-266, 1992; *Journal of Biotechnology* 25: 5 333-339, 1992; Hiroi *et al.*, 1976, *Svensk Papperstidning* 5: 162-166.

Oxidation of dyes or dye precursors and other chromophoric compounds leads to decolorization of the compounds. Laccase can be used for this purpose, which can be particularly advantageous in a situation in which a dye transfer between fabrics is undesirable, e.g., in the textile industry and in the detergent industry. Methods for dye transfer inhibition and dye oxidation can be found in WO 92/01406, WO 92/18683, EP 10 0495836 and Calvo, 1991, *Mededelingen van de Faculteit Landbouwwetenschappen/Rijksuniversiteit Gent.* 56: 1565-1567; Tsujino *et al.*, 1991, *Journal of the Chemical Society* 42: 273-282; methods for the use of oxidation of dye and dye precursors 15 in hair coloring are found in U.S. application Serial No. 08/441,146 and 441,147, the contents of which are incorporated herein by reference.

The present laccase can also be used for the polymerization of phenolic or aniline compounds present in liquids. An example of such utility is the treatment of juices, such as apple juice, so that the laccase will accelerate a precipitation of the phenolic compounds present in the juice, thereby producing a more stable juice. Such applications have been 20 described in Stutz, 1993, *Fruit Processing* 7/93, 248-252; Maier *et al.*, 1990, *Dt. Lebensmittel-rindschau* 86: 137-142; Dietrich *et al.*, 1990, *Fluss. Obst* 57: 67-73.

The present invention is further explained in the following non-limiting examples.

### Examples

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#### Materials and methods

Chemicals used as buffers and substrates are commercial products of at least reagent grade.

The protocols for molecular biology experiments (including restriction digests, DNA 30 ligations, gel electrophoresis, and DNA preparations) are adapted from either the instructions of the manufacturer or standard procedures (Sambrook *et al.*, 1989, *supra*). All oligonucleotides are synthesized by an Applied Biosystems 294 DNA/RNA Synthesizer.

Nucleotide sequences are determined by an Applied Biosystems automatic DNA Sequencer, Model 373A, version 1.2.0.

**Example 1: Site-directed mutagenesis of *Myceliophthora thermophila* laccase**

5       The construction of a *Myceliophthora thermophila* laccase expression vector, pRaMB17, and several derivatives, pRaMB17M, pBANe22T, and pRaMB17Q, which direct expression of the *Myceliophthora thermophila* wild-type laccase and laccase variants, is shown in Figures 1-4. The primers used in the constructions are summarized in Table 1.

Table 1 - Primers

10	<u>Primer</u>	<u>Sequence</u>
1	(forward) 5' dTCGTCTACCTCGAGCGGCC 3'	(SEQ ID NO:1)
2	(reverse) 5' dTCATCTAGACGCTCACGCCTTGACCAGCCA 3'	(SEQ ID NO:2)
3	5' dTAGACGACGCCGAAGCCGCCCAGAC 3'	(SEQ ID NO:3)
4	5' dGACGACGCCAGGCCAGCCTCGAGGTGCCAGGCGATGTG 3'	(SEQ ID NO:4)
15	5' dGAGGTAGACGACGCCGAAGCCAGCCTCGAGGTGCCAGGCGATGTG 3'	(SEQ ID NO:5)
6	5' CGGTACCGTCTAGAGTCGCGATGCATC 3'	(SEQ ID NO:6)
7	3' CCGGGCCATGGCAGATCTCAGCGCTACGTAGGATC 5'	(SEQ ID NO:7)
8	5' ATGATGAAGTCCTTCATCAGCGCCGACGCTTTGGTGGG 3'	(SEQ ID NO:8)
9	3' TACTACTCAGGAAGTAGTCGCGCGCTGCAGAAAACCAC 5'	(SEQ ID NO:9)
20	10 (forward) 5' dGGGTCTAGAGGTGACTGACACCTGGCGGT 3'	(SEQ ID NO:10)
11	(reverse) 5' dTGACCCGGAACTGGCCCCGACATTCCAGC 3'	(SEQ ID NO:11)
12	5'-gggatttaatATGAAGTCCTTCATCAGCGCC-3'	(SEQ ID NO:12)
13	5'-gggttaattaaTtACGCCTTGACCAGCCACTCGCC-3'	(SEQ ID NO:13)
14	5' ATACACAACGGATGATGAAGTCCTTCATCAGCG 3'	(SEQ ID NO:14)
25	Specifically, a small DNA fragment containing the 3'-terminus of the <i>lcc-1</i> coding region (including stop codon) is generated by PCR using pRaMB5 (U.S. application Serial No. 08/441,146, which is incorporated herein by reference) as a template for <i>Pfu</i> polymerase with primers 1 and 2 listed in Table 1. The 188 bp PCR product is digested with <i>Xba</i> I plus <i>Xho</i> I and purified by agarose gel electrophoresis. The purified fragment is then mixed in a three-part ligation reaction with an <i>Asp</i> 718I- <i>Xho</i> I segment (1286 bp) of the <i>lcc-1</i> gene from pRaMB5, and pUC518 (a derivative of pUC118; Vieira and Messing, 1987, <i>Methods in Enzymology</i> 153: 3-4), containing additional restriction sites for <i>Bgl</i> II, <i>Cla</i> I, <i>Xho</i> I and <i>Nsi</i> I in the polylinker, which has been cleaved with <i>Asp</i> 718I- <i>Xba</i> I. The resulting plasmid, pInt2.22, which contains approximately 1.5 kb of the <i>lcc-1</i> coding region, is extended from an internal <i>Asp</i> 718I site through the stop codon which is followed immediately by a <i>Xba</i> I site. Single-stranded pInt2.22 DNA template is prepared (Vieira and Messing, 1987, <i>supra</i> ) and	
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used as a template for oligonucleotide-directed mutagenesis (Adelman *et al.*, 1983, *DNA* 2: 183-193) with primer 3 for L513F mutation, primer 4 for V509L/S510E/G511A mutation, and primer 5 for V509L/S510E/G511A/L513F mutation to derive the precursor plasmids for pRaMB17, pRaMB17M, pBANe22T and pRaMB17Q.

5        Mutants are identified by hybridization with radiolabeled oligonucleotide primers 3, 4, and 5, and each mutation is verified by DNA sequence analysis.

10      The next step in the construction of pRaMB17 and its derivatives is partially shown in Figure 2. The starting plasmid, pMWR3-SAN, is prepared by cleaving bacteriophage vector M13mp18 (Yanisch-Perron *et al.*, 1985, *Gene* 33: 103-119) with *Hind*III and *Eco*RI, and purifying the large vector fragment by agarose gel electrophoresis. This fragment is ligated with a synthetic DNA linker having the following sequence:

5' AATTCTCGACGGYCTCTATTCTGTACGGCCTTCAGGTGGCCGCACCGGCCA  
TGCATAGCAGCTGCCAGAGATAAAGACATGCCGGAAGTCCACCGGCGTGGCCG  
GTACGTATTCGA 3' (SEQ ID NO:15)

15      The resulting phage vector, mp18-5'link, is then digested with *SaII* and *BsaI* (both sites in the synthetic linker region) and ligated with a 1.1 kb *SaII-BsaI* fragment from pTAKA-17 comprising the TAKA promoter region to generate the recombinant phage mp18-5'. Plasmid pUC18 (Yanisch-Perron *et al.*, 1985, *supra*) is digested with *Hind*III plus *Eco*RI and the 2.6 kb vector fragment is purified by agarose gel electrophoresis. The isolated fragment is ligated with a synthetic linker with the following sequence:

5' AATTGTTAAACTCTAGAGAATTCAAGCTTGTGACGTTAAACCAAATT  
GAGATCTCTTAAGTTCGAACAGCTGCAAATTGTCGA 3' (SEQ ID NO:16)

20      The resulting plasmid, pUC18::TAKA-link, is digested with *SaII* plus *Eco*RI and the vector fragment is isolated by agarose gel electrophoresis. pTAKA-17 is used as a template for PCR amplification of a 0.7 kb TAKA-amylase terminator fragment. For this purpose, the following primers are used:

forward primer: 5' dATGCATAGGGTGGAGAGTATGATGG 3' (SEQ ID NO:17)  
reverse primer: 5' dCTGAATTCCGTTTCGTTAC 3' (SEQ ID NO:18)

25      The 0.7 kb product of this PCR reaction is digested with *Nsi*I plus *Eco*RI and mixed in a three-part ligation with *SaII* and *Eco*RI cleaved pUC18::TAKA-link and the 1.1 kb *SaII-Nsi*I TAKA promoter fragment from mp18-5' to produce pMWR1.

Plasmid pMWRI is modified to generate pMWR3. First, a new TAKA-amylase promoter segment is generated by PCR using pTAKA-17 as a template with the following synthetic primers:

forward primer: 5' dTCCTGCAGAATGCAATTAAACTC 3' (SEQ ID NO:19)

5 reverse primer: 5' dCTATGCATATTAAATGCCTCTGTGGGGTTATTG 3' (SEQ ID NO:20)

The 0.2 kb PCR product is digested with *Nsi*I plus *Pst*I and ligated with the large vector fragment of pMWRI which has been cleaved with *Nsi*I and *Pst*I. The resulting plasmid, pMWR3, is then modified by inserting a small linker, AATTGGGCCATGCA (SEQ ID NO:21), which contains an *Apa*I site between the *Swa*I and *Nsi*I sites, creating pMWR3-SAN. A derivative of pMWR3-SAN is then constructed by replacing the *Apa*I-*Xba*I TAKA-amylase terminator fragment with a small linker (primers 6 and 7 shown in Table 1). This linker introduces *Asp*718I, *Xba*I, and *Nru*I cloning sites and inactivates the *Xba*I site of pMWR3-SAN yielding pMWR3L.

15 pMWR3L is digested with *Swa*I and *Asp*718I and mixed in a three-part ligation with a 853 bp *Bsm*I-*Asp*718I fragment comprising the 5'-end of the *lcc-1* coding region and synthetic DNA adapter containing the translation initiation region (primers 8 and 9 shown in Table 1) to yield plasmid pInt1.

20 A 597 bp DNA segment comprising the *Aspergillus niger glaA* terminator region is then isolated by PCR using pHD414 (EP 238 023) as a template with primers 10 and 11 shown in Table 1, which introduce *Xba*I and *Sma*I sites at the 5' and 3'-ends of the terminator, respectively. The amplified DNA fragment is subsequently cleaved with *Xba*I plus *Sma*I and subcloned into pUC118 to generate plasmid pUC::AMGterm.

25 Finally, the 1.5 kb fragments containing the wild-type and mutant *lcc-1* gene sequences are excised by digestion with *Asp*718I and *Xba*I and purified by agarose gel electrophoresis. Each of these fragments is mixed in a three part ligation (Figure 3) with *Asp*718I and *Nru*I digested pInt1 plus the 597 bp *Xba*I-*Sma*I *glaA* terminator fragment from pUC::AMGterm to produce pRaMB17, pRaMB17M, pRaMB17T and pRaMB17Q.

30 DNA primers 12 and 13 (uppercase letters represent sequences in the laccase gene) are used in a PCR reaction to amplify the mutant laccase gene from plasmid pRaMB17T (Figure 4). The PCR is performed in a 50 ml reaction containing 120 ng of plasmid pRaMB17T, 0.05 mM each of dATP, dTTP, dGTP, dCTP, 100 pmol each of primers 12 and

13, 1X *Pwo*I Buffer (Boehringer Mannheim, Indianapolis, IN), 5% (v/v) DMSO, and 2.5 units *Pwo*I (Boehringer Mannheim, Indianapolis, IN). The PCR conditions are 95°C for 3 minutes, 30 cycles each at 95°C for 1 minute, 60°C for 1 minute, and 72°C for 1.5 minutes, and then 72°C for 5 minutes. The PCR reaction mixture is run on a agarose gel and the 2.4 kb DNA laccase band is excised. The DNA is purified by solubilization of the agarose with 5 3 volumes Qia-ex solubilization buffer (Qiagen, Los Angeles, CA) followed by a Qiaquick PCR spin column according to the manufacturer's directions (Qiagen, Los Angeles, CA). The DNA is recovered in 50 ml of 1 mM EDTA-10 mM Tris pH 8 buffer. A 20 µl aliquot of the DNA is cut in a final volume of 25 µl containing 1X restriction enzyme buffers and 10 restriction enzymes *Pac*I and *Swa*I as suggested by the manufacturer. The reaction mixture is then heated at 80°C for 10 minutes. One ml of the *Pac*I/*Swa*I cut laccase gene is ligated into *Pac*I/*Swa*I cut plasmid pBANe6. The ligation mixture is used to transform *E. coli* strain DH5 $\alpha$ . The plasmid containing pBANe6 and the mutant laccase sequences is designated 15 pJeRS31. pJeRS31 is subjected to site-directed mutagenesis using primer 14 to remove the *Swa*I site and add a second ATG using the MORPH Site-Specific Plasmid DNA Mutagenesis Kit according to the manufacturer's instructions (5 Prime 3 Prime, Inc., Boulder, CO) to produce pBANe22T.

A summary of the plasmids is provided in Table 2.

Table 2. pRaMB17 and its derivatives

20	<u>Vector</u>	<u>MtL protein encoded</u>
	pRaMB17	Wild-type MtL
	pRaMB17M	MtL with the L513F mutation
	pBANe22T	MtL with the triple substitution V509L/S510E/G511A
25	pRaMB17Q	MtL with the quadruple substitution V509L/S510E/G511A/L513F

Example 2: Transformation of *Aspergillus oryzae* with modified *Myceliophthora thermophila* laccase genes

Methods for co-transformation of *Aspergillus oryzae* are described by Christensen *et al.*, 1988, *Bio/Technology* 6: 1419-1422. For introduction of each of the *Myceliophthora thermophila* laccase expression vectors pRaMB17, pRaMB17M, pBANe22T, and pRaMB17Q 30 into *Aspergillus oryzae* HowB711, equal amounts (approximately 5 µg each) of the laccase expression vector and pToC90 (WO 91/17243) are added to approximately 106 protoplasts

in suspension while pBANe22T is added alone. Transformants are selected on Cove medium (Cove, 1966, *Biochimica Biophysica Acta* 113: 51-56) containing 1 M sucrose, 10 mM acetamide as the sole nitrogen source, and 20 mM CsCl to inhibit background growth. The transformants selected in this way are subsequently screened for the ability to produce laccase on Cove medium containing 1-3 mM ABTS. Cells which secrete active laccase oxidize the ABTS, producing a green halo surrounding the colony. Transformants which produce detectable laccase activity on ABTS plates are purified twice through conidiospores.

5 **Example 3: Expression of modified *Myceliophthora thermophila* laccases**

10 The transformants described in Example 2 are grown in shake flask cultures containing 25 ml of ASPO4 medium (pRaMB17, pRaMB17M, pRaMB17Q) or MY51 medium (pBANe22T) for 4 to 5 days at 37°C. ASPO4 medium is comprised of 1 g of CaCl<sub>2</sub>-2H<sub>2</sub>O, 2 g of yeast extract, 1 g of MgSO<sub>4</sub>, 2 g of citric acid, 5 g of KH<sub>2</sub>PO<sub>4</sub>, 1 g of urea, 2 g of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 20 g of maltodextrin, and 0.5 ml of trace metals solution per liter.

15 MY51 medium is comprised of 50 g of maltodextrin, 2 g MgSO<sub>4</sub>-7H<sub>2</sub>O, 10 g of KH<sub>2</sub>PO<sub>4</sub>, 2 g of citric acid, 10 g of yeast extract, 2 g of urea, 1 g of urea, 2 g of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, and 0.5 ml of trace metals solution. The trace metals solution is comprised of 14.3 g of ZnSO<sub>4</sub>-7H<sub>2</sub>O, 2.5 g of CuSO<sub>4</sub>-5H<sub>2</sub>O, 0.5 g of NiCl<sub>2</sub>-6H<sub>2</sub>O, 13.8 g of FeSO<sub>4</sub>-7H<sub>2</sub>O, 8.5 g of MgSO<sub>4</sub>-H<sub>2</sub>O, and 3.0 g of citric acid per liter of RO water. Culture supernatants are assayed for

20 laccase activity using either ABTS or syringaldazine as a substrate as described below.

Syringaldazine (SGZ) oxidation is determined in MES pH 5.3 buffer or Britten-Robinson buffer, pH 2.7 to 11.0, with 10% ethanol (coming from SGZ stock solution) by monitoring the absorbance change at 530 nm with an extinction coefficient of 65 mM<sup>-1</sup>cm<sup>-1</sup> (Bauer and Rupe, 1971, *Analytical Chemistry* 43: 421-425) at 20°C. Laccase activity using SGZ as a substrate is assayed by mixing 800 µl of assay buffer (40 µM CuSO<sub>4</sub>-25 mM sodium acetate pH 5.5) with 20 µl of culture supernatant and 60 µl of 0.28 mM syringaldazine in 50% ethanol. The absorbance at 530 nm is measured over time in a UV-VIS spectrophotometer. One laccase unit (LACU) is defined as the amount of enzyme which oxidizes one µmole of substrate per minute at 30°C.

30 ABTS oxidation is determined at pH 5 in a 96-well plate at 20°C by monitoring the absorbance change at 405 nm with an extinction coefficient of 35 mM<sup>-1</sup>cm<sup>-1</sup> (Childs and Bardsley, 1975, *Biochemical Journal* 145: 93-103). Laccase activity using ABTS as a

substrate is measured by mixing 20  $\mu$ l of culture supernatant with 200  $\mu$ l of substrate solution containing 0.275 mg of ABTS per ml of 100 mM sodium acetate pH 5.0.

Shake flask cultures producing high levels of extracellular laccase activity are further evaluated by fermentation. A 1 ml aliquot of a spore suspension (approximately 10<sup>9</sup> spores) of an *Aspergillus oryzae* transformant expressing the laccase variant of interest is added aseptically to each of several 500 ml shake flasks containing 100 ml of medium comprised of 50 g of Nutriose 725, 2 g of MgSO<sub>4</sub>·7H<sub>2</sub>O, 10 g of KH<sub>2</sub>PO<sub>4</sub>, 2 g of K<sub>2</sub>SO<sub>4</sub>, 0.5 g of CaCl<sub>2</sub>·2H<sub>2</sub>O, 2 g of citric acid, 10 g of yeast extract, 0.5 ml of trace metals (as described above), and 2 g of urea per liter of tap water (adjusted to pH 6.0 before autoclaving) and incubated at 34°C on a rotary shaker at 200 rpm for about 18 hours. Samples of the shake flask broths are then transferred to a laboratory fermentor containing medium, supplemented with 2 mM CuSO<sub>4</sub>·5H<sub>2</sub>O, comprised of 30 g of Nutriose, 5 g of yeast extract, 2 g of (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub>, 2 g of MgSO<sub>4</sub>·7H<sub>2</sub>O, 4 g of citric acid, 3 g of K<sub>2</sub>SO<sub>4</sub>, 2 g of CaCl<sub>2</sub>·H<sub>2</sub>O, and 0.5 ml of trace metals solution (as described above) per liter and fed during the course of the fermentation with a medium comprised of 270 g of Nutriose, 30 g of urea, and 15 g of yeast extract per liter. The fermentaion is allowed to proceed at 31°C, pH 7, 600-700 rpm for 7 days.

Laccase yields for the "M" (L513F) and "T" (V509L/S510E/G511A) mutants from these fermentations are estimated to be 25% and 40%, respectively, of the wild-type yield. In contrast, the expression yield of mutant "Q" (V509L/S510E/G511A/L513F) is so low that there is insufficient laccase for purification.

#### Example 4: Purification of modified *Myceliophthora thermophila* laccases

The wild-type, "M", and "T" fermentation broths from Example 3 are cheese-cloth filtered (pH 7.6, 16 mS), filtered through Whatman #2 filter paper, concentrated on a Spiral Concentrator (Amicon) with a S1Y100 membrane (100 kDa MW-CO), and diluted to 0.75 mS with glass distilled water. The washed concentrated broths are loaded onto a Q-Sepharose XK26 (Pharmacia, Uppsala, Sweden) column (120 ml), pre-equilibrated with 10 mM Tris, pH 7.5, 0.7 mS (Buffer A), and active fractions are eluted during the linear gradient with Buffer B (Buffer A plus 2 M NaCl). The active fractions are pooled, adjusted to 1 mS in ionic strength, and subjected to a Mono-Q (Pharmacia, Uppsala, Sweden)

chromatography equilibrated with Buffer A. Laccase preparations with apparent electrophoretic purity are obtained in the run-through fractions.

**Example 5: Site-directed mutagenesis of *Rhizoctonia solani* laccase gene**

Site-specific mutations are introduced into the *Rhizoctonia solani* laccase *rsl4* gene of the expression plasmid, pJiWa59, using the overlap-extension PCR method (Ho, 1989, *Gene* 77: 51-59) together with the primers listed in Table 3. Primer 15 (SEQ ID NO:22) is used to create pJiWa85 that encodes three amino acid changes ("T": L466V/E467S/A468G) in the laccase coding region (Table 3). PCR amplification with primer 16 (SEQ ID NO:23) results in pJiWa86 which encodes a single amino acid mutation ("M": L470F). For each mutation, a 505 nt *SacI/NorI* fragment is generated by PCR and used to replace the homologous fragment in pJiWa59. PCR-amplified regions of the gene are sequenced to confirm the mutation as well as to ascertain the integrity of the coding region.

Table 3. Primers used for PCR mutagenesis of *rsl4* gene.

Primer 15	C ATT GAC TGG CAC <u>G</u> TG <u>T</u> CG <u>G</u> GT GGG CTC GCA CTT G
pJiWa85	<i>I</i> D W H V S G G L A L
	: : : :
RsL-wt	H I D W H L E A G L A L V
<i>rsl4</i>	CAC ATT GAC TGG CAC TTG GAG GCT GGG CTC GCA CTT GTC
	: : : :
pJiWa86	<i>L</i> E A G F A L V
Primer 16	C TTG GAG GCT GGG <u>T</u> TC GCA CTT GTC

Note. The amino acid translation of both the primers and the native gene are shown in italics. Homologous amino acids are noted by a colon between the two sequences. Those nucleotides in the PCR primers which differ from the gene sequence are underlined.

**Example 6: Transformation of *Aspergillus oryzae* with the modified *Rhizoctonia solani* *rsl4* genes**

*Aspergillus oryzae* HowB711 is transformed with 8 µg of pJiWa85 ("T": L466V/E467S/A468G) or pJiWa86 ("M": L470F) together with 2 µg of pToC90 and *Aspergillus oryzae* HowB104 is transformed with 8 µg of pJiWa59 (wt) together with 2 µg

of pToC90 using a standard PEG mediated protocol (Yelton, 1984, *Proceedings of the National Academy of Sciences USA* 81: 1470-1474). The transformants are selected on Minimal medium plates supplemented with 10 mM acetamide and 1 M sucrose. The Minimal medium is comprised of 6.0 g of NaNO<sub>3</sub>, 0.52 g of KCl, 1.52 g of KH<sub>2</sub>PO<sub>4</sub>, 1.0 ml of trace metals solution, 20 g of Nobel Agar (Difco), 20 ml of 50% glucose, 20 ml of methionine (50 g/l), 20 ml of biotin (200 mg/l), 2.5 ml of 20% MgSO<sub>4</sub>-7H<sub>2</sub>O, and 1.0 ml of mg/ml streptomycin per liter. The agar medium is adjusted to pH 6.5 prior to autoclaving and then glucose, methionine, biotin, MgSO<sub>4</sub>-7H<sub>2</sub>O, and streptomycin are added as sterile solutions to the cooled autoclaved medium and poured into plates. The trace metals solution is comprised of 0.04 g of Na<sub>2</sub>B<sub>4</sub>O<sub>7</sub>-10H<sub>2</sub>O, 0.4 g of CuSO<sub>4</sub>-5H<sub>2</sub>O, 1.2 g of FeSO<sub>4</sub>-7H<sub>2</sub>O, 0.7 g of MnSO<sub>4</sub>-H<sub>2</sub>O, 0.8 g of Na<sub>2</sub>MoO<sub>2</sub>-2H<sub>2</sub>O, and 10 g of ZnSO<sub>4</sub>-7H<sub>2</sub>O per liter of RO water.

Laccase activity is scored on Minimal medium plates containing 10 mM acetamide and 1 g/l ABTS. Colonies that produce a green halo, indicative of laccase expression, are spore-purified twice.

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#### Example 7: Expression of modified *Rhizoctonia solani* laccases

The spores from transformants of pJiWa59 (wt), pJiWa85 ("T"), and pJiWa86 ("M") described in Example 6 are used to inoculate 15 ml of MY51 medium in 125 ml shake flasks. After 3 days and 5 days growth at 37°C, a 1 ml aliquot is removed from each shake flask and centrifuged at 14,000 g for 5 minutes to remove any mycelia clumps. The supernatants are assayed for ABTS oxidation in 96-well microtiter plates as described below.

ABTS oxidation is determined in MES pH 5.3 buffer or Britten-Robinson buffer at pH 2.7 to 11.0 in a 96-well plate at 20°C by monitoring the absorbance change at 405 nm with an extinction coefficient of 35 mM<sup>-1</sup>cm<sup>-1</sup> (Childs and Bardsley, 1975, *Biochemical Journal* 145: 93-103).

The transformants yielding the highest laccase activity are selected for fermentation and grown as described in Example 3. Laccase yields for the "M" (L470F) and "T" (L466V/E467S/A468G) mutants from these fermentations are estimated to be 17% and 50%, respectively, of the wild-type yield.

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#### Example 8: Purification of *Rhizoctonia solani* modified laccases

The wild-type, "M", and "T" fermentation broths from Example 7 are cheese-cloth filtered (pH 7.6, 16 mS), filtered through Whatman #2 filter paper, and concentrated on a Spiral Concentrator (Amicon) with a S1Y100 membrane (100 kDa MW-CO). The concentrated broths are then applied to a Q Sepharose column (XK26, 120 ml) (Pharmacia, Uppsala, Sweden), preequilibrated with 10 mM Tris pH 7.5, 0.7 mS (Buffer A). Active fractions run through the column during loading and washing. The active fractions are pooled, adjusted to pH 5.3 and applied on a SP-Sepharose column (XK16, 60 ml) (Pharmacia, Uppsala, Sweden), preequilibrated with 10 mM MES pH 5.3 buffer (Buffer C). The majority of activity is eluted by a linear gradient of Buffer D (Buffer C and 1 mM NaCl). The active fractions are adjusted to 20 mS and applied to a Sephadex 200 column (1610, 120 ml) (Pharmacia, Uppsala, Sweden), pre-equilibrated with Buffer E (Buffer C and 0.1 M NaCl). Purified *Rhizoctonia solani* laccase fractions are eluted by Buffer E. A recovery of 1% or 5% and purification of 280- or 150-fold are achieved for mutants "M" and "T", respectively. The "T" mutant shows a three-fold higher yield than the "M" mutant, but two-fold lower yield than the wild-type laccase.

**Example 9: Characterization of the modified *Myceliophthora thermophila* laccases and the *Rhizoctonia solani* modified laccases**

The Leu/Phe mutation causes a decrease in expression yield. RsL-"M" shows a yield which is three-fold lower than that of RsL-"T", and five-fold lower than that previously obtained for RsL-wild type; while MtL-"M" shows a yield approximately two-fold lower than that observed for MtL-"T" and five-fold lower than that observed for MtL-wild type. When *Polyporus pinsitus* laccase (PpL; U.S. application Serial No. 08/441,147, which is incorporated herein by reference), *Rhizoctonia solani* laccase (including isozyme 1 and 3; WO 95/07988), MtL, *Scytalidium thermophilum* laccase (StL), and *Myrothecium verrucaria* bilirubin oxidase (BiO) are expressed in the same host (HowB104), the yields are in the order of BiO ~ MtL ~ StL > RsL-4 > RsL-1 ~ PpL. Among these laccases, the residue corresponding to the modified Leu in the "M" mutants is: Phe for both PpL and RsL-1; Leu for RsL-4, MtL, and StL; and Met for BiO. It seems that a Phe at this particular position correlates to low expression yield of these laccases in *Aspergillus oryzae* HowB104 and HowB711 strains.

The triple mutations in RsL-mutant "T" (LEA -> VSG), which eliminates the negative charge, decreases activity two orders of magnitude. The triple mutations in MtL-mutant "T" (VSG -> LEA), which creates a negative charge, decreases activity 4-fold. In contrast, the "M" mutants, in which a Leu is replaced by a Phe, exhibit similar activity in comparison to their wild type counterparts. The *Rhizoctonia solani* results are consistent with the hypothesis which correlates the presence of negative charge(s) near the T1 Cu to the specific activity. The effect of the Glu in the selected pentapeptide segment could be attributed to general base-catalysis in which the negatively charged residue facilitates the electron transfer from the substrate to the T1 Cu by perturbing the substrate molecule and/or stabilizing the resulting electron-deficient intermediate or product molecule.

The molecular weights of the mature laccases are used to calculate both extinction coefficients and turnover numbers. The molecular weights are determined from the deduced amino acid sequences of the DNA sequences (Figure 6: SEQ ID NOS:24 and 25, and Figure 7: SEQ ID NOS:26 and 27). Protein concentrations (expressed as subunits) are measured based on the extinction coefficients determined by quantitative amino acid analysis.

Cyclic voltammetry measurements with a Pt electrode show a mid-potential of 0.76 V for Fe(dipyridyl)<sub>2</sub>Cl<sub>3</sub> - Fe(dipyridyl)<sub>2</sub>Cl<sub>2</sub> couple in 8 mM MES pH 5.3. The oxidation of ABTS and SGZ in 8 mM MES pH 5.3 yields a mid-potential of 0.70 and 0.63 V, respectively. The published redox potentials ( $E^\circ$ ) for the redox couples Fe(dipyridyl)<sub>2</sub>Cl<sub>3</sub> - Fe(dipyridyl)<sub>2</sub>Cl<sub>2</sub>, NaI<sub>3</sub> - NaI, and K<sub>3</sub>Fe(CN)<sub>6</sub> - K<sub>4</sub>Fe(CN)<sub>6</sub> are 0.780, 0.536, and 0.433 V, respectively (Kolthoff and Tomsicek, 1936, *Journal of Physical Chemistry* 40: 247-255; O'Reilly, 1973, *Biochimica Biophysica Acta* 292: 509-515; Vanysek, 1992, In Lide, D. R., editor, *Handbook of Chemistry and Physics*, 73rd Edition, pages 8.17-8.22, CRC Press, Boca Raton, Florida). The  $E^\circ$  determination for *Rhizoctonia solani* laccase is performed in 8 mM MES pH 5.3 buffer with either 17  $\mu$ M *Rhizoctonia solani* laccase, 0.2 mM Fe(bipyridyl)<sub>2</sub>Cl<sub>2</sub>, and 0.05 - 0.2 mM Fe(bipyridyl)<sub>2</sub>Cl<sub>3</sub>, or 71 - 78  $\mu$ M *Rhizoctonia solani* laccase and 14 - 100  $\mu$ M ABTS. The  $E^\circ$  determination for MtL is performed in 8 mM MES, pH 5.3 with 0.14 mM MtL, 0.02 - 20 mM K<sub>3</sub>Fe(CN)<sub>6</sub>, and 2 mM K<sub>4</sub>Fe(CN)<sub>6</sub>; as well as with 31  $\mu$ M MtL, 0.1 mM I<sub>2</sub>, and 5 - 19 mM NaI. Britten-Robinson buffer is used for other pHs. Under various potentials of the solution poised by various concentration ratios of the redox couples, the absorbance changes of laccase in the range of 550-800 nm are monitored and the concentrations of the oxidized copper (II) and reduced copper (I) states are calculated after

the spectral change reaches equilibrium. The concentrations of the redox couples at equilibrium are calculated from the initial concentrations and the concentration changes caused by the interaction with laccase. In the case of measuring  $E^\circ$  of *Rhizoctonia solani* laccase with ABTS, the concentration of ABTS cation radical ( $ABTS^+$ ) is measured by the absorption at 810 nm (where *Rhizoctonia solani* laccase has no contribution) and then the spectral contribution of  $ABTS^+$  at 600 nm is subtracted from the observed absorption value in order to assess the spectral change of *Rhizoctonia solani* laccase. Anaerobicity is achieved by repetitive evacuating and argon-flushing the reaction chamber at 4°C.

The mutants exhibit similar chromatographic elution patterns to their wild type counterparts. The purified preparations have a characteristic blue color typical of a laccase and show other typical laccase properties as shown in Table 4. All the mutants can be retained by a 100 kDa MW-CO membrane, indicating a dimeric nature.

Table 4. Properties of *Myceliophthora thermophila* and *Rhizoctonia solani* laccase mutants

		MW*, kDa	$\lambda_{max} (\epsilon)^\dagger$	$E^\circ$ at pH 5.3‡
15	pJiWa59 (wt)	70-85	276 (66), 330sh (4.6), 602 (4.7)	0.73 ± 0.02
pJiWa86 ("M")	70-90	276 (63), 330sh (2.6), 600 (3.7)	0.72 ± 0.02	
pJiWa85 ("T")	70-90	276 (63), 330sh (1.7), 600 (4.8)	0.74 ± 0.03	
pRaMB17 (wt)	75-90	276 (134), 330sh (8.4), 589 (4.2)	0.47 ± 0.01	
20	pRaMB17M ("M")	70-90	280 (134), 330sh (6.1), 600 (3.8)	0.50 ± 0.01
pBANe22T ("T")	70-90	276 (134), 330sh (4.2), 600 (2.9)	ND□	

\* Estimated on SDS-PAGE. † Units:  $\lambda_{max}$ , nm;  $\epsilon$ ,  $mM^{-1}cm^{-1}$ . Calculated extinction coefficients are used. ‡ in V vs NHE. □Not determined.

25       $K_m$  and  $k_{cat}$  are obtained from the initial rate ( $v$ ), enzyme concentration ( $E$ ), and substrate concentration ( $S$ ) in accordance to the equation  $v = k_{cat} ES / (K_m + S)$  by non-linear regression fitting using the Prism program (GraphPad, San Diego, CA). The  $K_m$  and  $k_{cat}$  for ABTS and SGZ are measured spectroscopically in 8 mM MES-NaOH buffer, pH 5.3; while the values for other substrates are measured by oxygen electrode in Britten-Robinson buffer, pH 5.1 with a Hansatech DW1/AD device (Norfolk, England), with 0.4 - 4  $\mu$ M laccase in

0.3 - 0.5 ml Britten-Robinson buffer. The O<sub>2</sub> concentration in air-saturated buffer solution is assumed as the same in plain water (0.28 mM).

Tables 5 and 6 summarize the SGZ and ABTS oxidase activities of the mutants. For both *Rhizoctonia solani* laccase and *Myceliophthora thermophila* laccase, more profound difference is observed on the mutant "T" than that on the mutant "M" in comparison with the wild type. Figure 5 shows the pH-activity profiles of the mutants with ABTS and SGZ. For ABTS oxidation, a significant change is seen with RsL- "T", MtL- "M", and MtL- "T". The optimal pH of RsL- "T" is shifted  $\geq$  1 unit in comparison with the wild type laccase. For SGZ oxidation, an optimal pH at 7 is observed for MtL- "T", in contrast to the range of 5-7 for the wild type laccase. In terms of pH profile, the elimination of the negative charge in RsL- "T" induces a shift of the optimal pH in the acidic direction for SGZ oxidation, probably due to the reduced acidity at the T1 site caused by the Glu removal. The creation of a negative charge in MtL- "T" induces a shift of the optimal pH for activity on the alkaline direction, which could be attributed to the increased acidity at MtL's T1 site caused by the creation of the negative charge.

Table 5. Syringaldazine oxidase activity of the mutants

	LACU*	SOU† (pH <sub>op</sub> )
RsL wt	4.3	11 (7)
RsL "M"	2.2	4.7 (6)
RsL "T"	0.024	0.048 (7)
MtL wt	42	35 (6)
MtL "M"	24	25 (6)
MtL "T"	2	10 (7)

Activity unit:  $\mu\text{mol min}^{-1} \text{mg}^{-1}$ . \*25 mM sodium acetate pH 5.5, 30°C. † B&R buffer, 20°C, at optimal pH (value in parenthesis).

**Table 6. Syringaldazine and ABTS oxidase activity of the mutants**

		SGZ		ABTS	
		K <sub>m</sub> , μM	k <sub>cat</sub> , min <sup>-1</sup>	K <sub>m</sub> , μM	k <sub>cat</sub> , min <sup>-1</sup>
	RsL wt	28 ± 4	550 ± 40	52 ± 6	2500 ± 100
5	RsL "M"	35 ± 4	255 ± 11	125 ± 13	760 ± 30
	RsL "T"	3.9 ± 0.3	1.1 ± 0.1	60 ± 4	20 ± 1
	MtL wt	1.4 ± 0.2	4500 ± 200	110 ± 20	3800 ± 300
	MtL "M"	1.8 ± 0.2	3300 ± 100	43 ± 3	1800 ± 100
	MtL: "T"	0.9 ± 0.2	360 ± 20	11 ± 2	530 ± 20

**Claims****What is claimed is:**

- 5      1. A mutant of a blue copper oxidase, comprising a mutation selected from the group consisting of (a) a substitution of one or more amino acid residues with other amino acid residues, (b) an insertion of one or more amino acid residues and/or (c) a deletion of one or more amino acid residues, wherein the mutation is carried out at a position which is located no greater than 15Å from a Type I copper site.
- 10     2. A mutant according to claim 1, wherein the position is located no greater than 12Å from a Type I copper site.
- 15     3. A mutant according to claim 2, wherein the position is located no greater than 10Å from a Type I copper site.
- 20     4. A mutant according to claim 3, wherein the position is located no greater than 8Å from a Type I copper site.
- 25     5. A mutant according to claim 4, wherein the position is located no greater than 6Å from a Type I copper site.
- 30     6. A mutant according to claim 5, wherein the position is located no greater than 4Å from a Type I copper site.
7. A mutant according to claim 6, wherein the position is located no greater than 2.5Å from a Type I copper site.
8. A mutant according to claim 7, wherein the position is adjacent to an amino acid residue which is a Type I copper site ligand.

9. A mutant according to claim 7, wherein the amino acid residue which is mutated is a Type I copper site ligand.

10. A mutant of claim 1 in which the oxidase is a bilirubin oxidase.

5

11. A mutant of claim 1 in which the oxidase is a bilirubin oxidase.a) A mutant of claim 1 in which the oxidase is a phenoxazinone synthase.

12. A mutant of claim 1 in which the oxidase is an ascorbate oxidase.

10

13. A mutant of claim 1 in which the oxidase is a ceruloplasmin.

14. A mutant of claim 1 in which the oxidase is a nitrite reductase.

15

15. A mutant of claim 1 in which the oxidase is a laccase.

16. A mutant of claim 15 in which the oxidase is a fungal laccase.

17. A mutant of claim 16 in which the oxidase is a *Rhizoctonia* laccase.

20

18. A mutant of claim 17 in which the *Rhizoctonia* laccase comprises the amino acid sequence 466LEAGL470.

19. A mutant of claim 16 in which the oxidase is a *Myceliophthora* laccase.

25

20. A mutant of claim 19 in which the *Myceliophthora* laccase comprises the amino acid sequence 509VSGGL513.

21. A mutant of claim 1 in which (a) a neutral amino acid residue is substituted with a negative amino acid residue or (b) a positive amino acid residue is substituted with a negative or neutral amino acid residue.

22. A mutant of claim 1 in which a phenylalanine is substituted with another amino acid residue.

23. A mutant of claim 22 in which the other amino acid residue is a leucine.

5

24. A mutant of claim 1 in which (a) a neutral amino acid residue is substituted with a positive amino acid residue or (b) a negative amino acid residue is substituted with a positive or neutral amino acid residue.

10 25. A mutant of claim 1 in which leucine or phenylalanine is substituted with a neutral residue selected from the group consisting of histidine, serine, threonine, tyrosine, cysteine, and methionine.

15 26. A mutant of claim 1 which is modified in a segment corresponding to 416VIELNITGGADHPI429 of *Rhizoctonia solani* laccase and 421ENDPGAPFTLPHM433 of *Myceliophthora thermophila* laccase.

20 27. A mutant of claim 1 which is modified in a segment corresponding to 474GPWFVHCHIDWHLEAGLVF494 of *Rhizoctonia solani* laccase and 497GAWLFHCHIAWHVSGGLGV515 of *Myceliophthora thermophila* laccase.

25 28. A mutant of claim 1 which is modified in a segment corresponding to 356VSLNLAIGRSTVDGIL371 of *Rhizoctonia solani* laccase and 361VTLDTTGTPLFWKVN376 of *Myceliophthora thermophila* laccase.

29. A mutant of claim 1 which is modified in a segment corresponding to 303LDPTNVFAVL312 of *Rhizoctonia solani* laccase and 308AIFHYAGAPG317 of *Myceliophthora thermophila* laccase.

30 30. A mutant of claim 1 which is modified in a segment corresponding to 217INVKRGKRYR226 of *Rhizoctonia solani* laccase and 222GRRHRLRLIN231 of *Myceliophthora thermophila* laccase.

31. A mutant of claim 1 which is modified in a segment corresponding to 465LEAGL472 of *Rhizoctonia solani* laccase.

5       32. A mutant of claim 1 which is modified in a segment corresponding to 466LEAGL470 of *Rhizoctonia solani* laccase.

33. A mutant of claim 1 which is modified by at least two amino acid residues.

10      34. A mutant of claim 33 which is modified by at least 3 amino acid residues.

35. A mutant according to claim 1, wherein the mutation is a substitution.

36. A nucleic acid construct comprising a nucleic acid sequence encoding the mutant of claim 1.

15      37. A host cell comprising the construct of claim 36.

20      38. A method for producing a mutant of a blue copper oxidase, comprising culturing a host cell of claim 37 under conditions conducive to expression of the mutant and recovering the mutant.

1/15

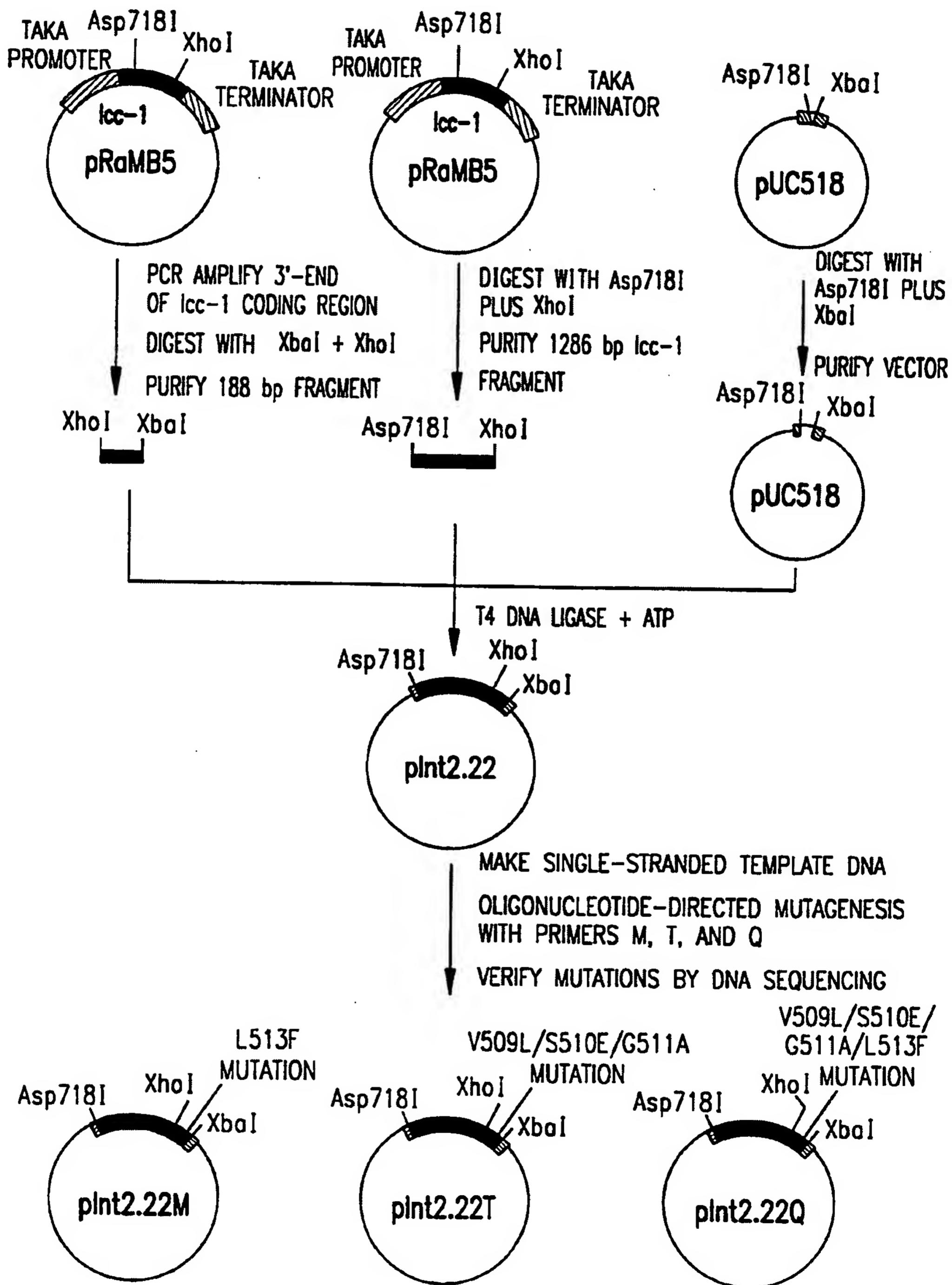


FIG. 1

2/15

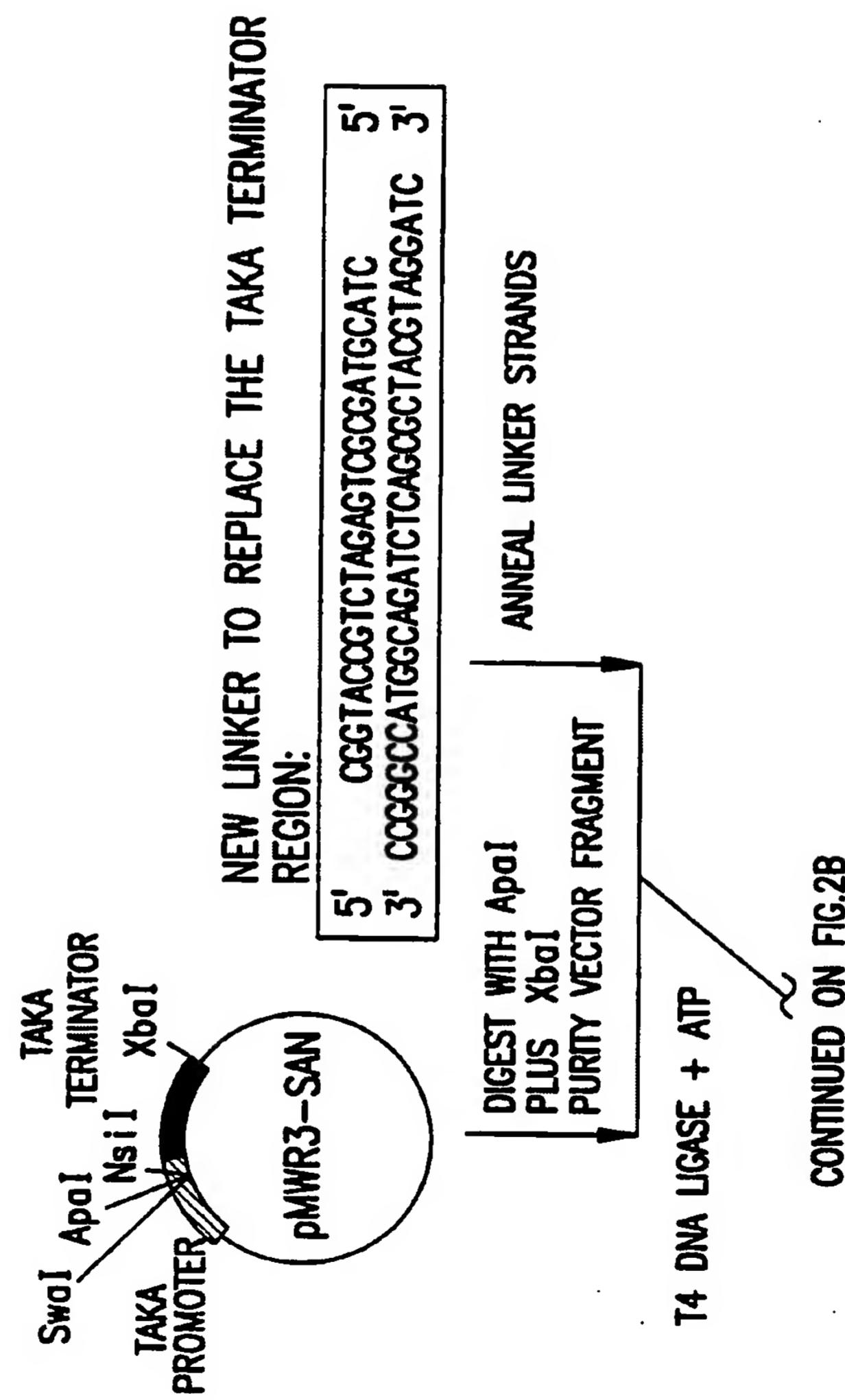
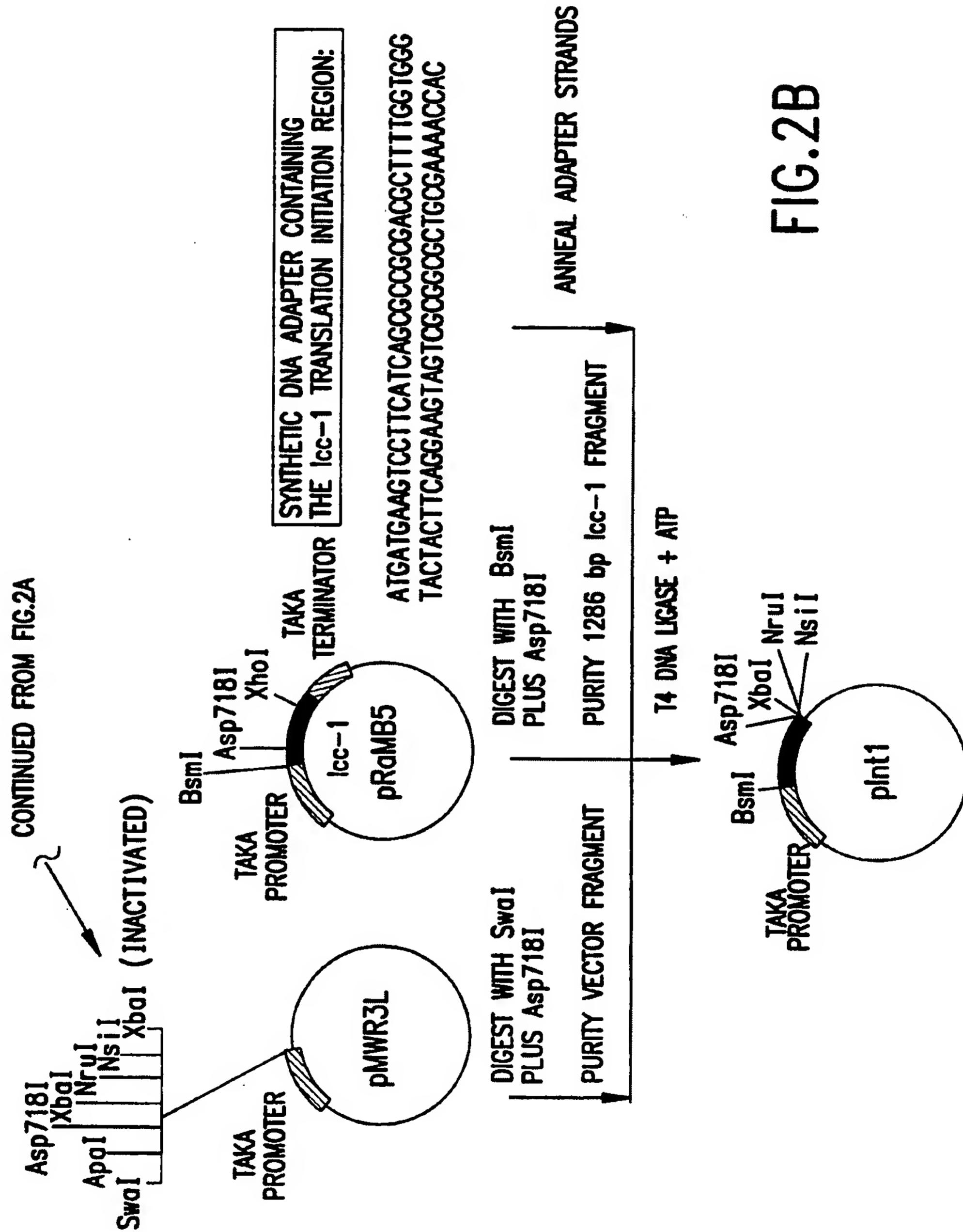


FIG.2A

3/15

**FIG.2B**

4/15

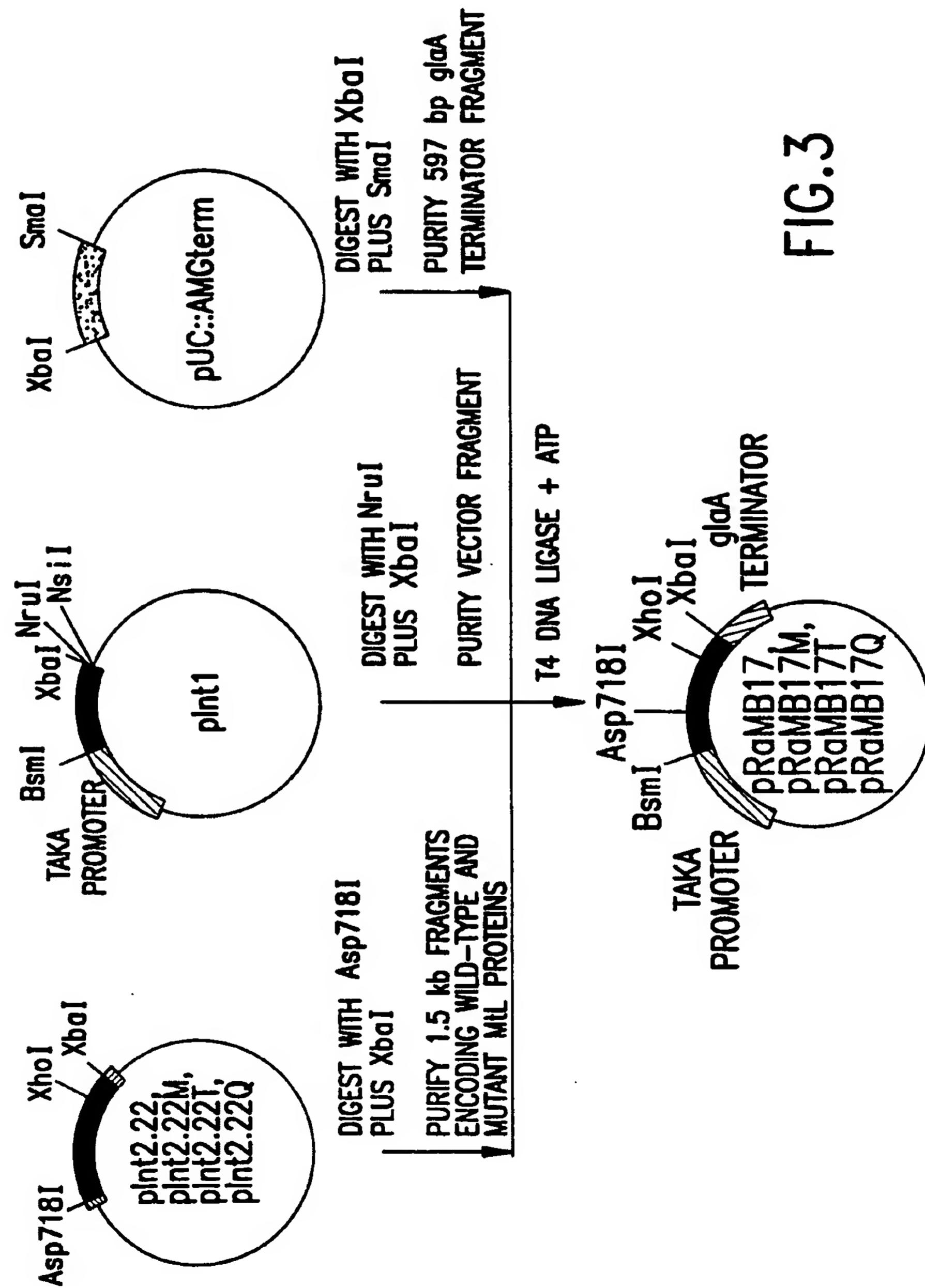


FIG. 3

5/15

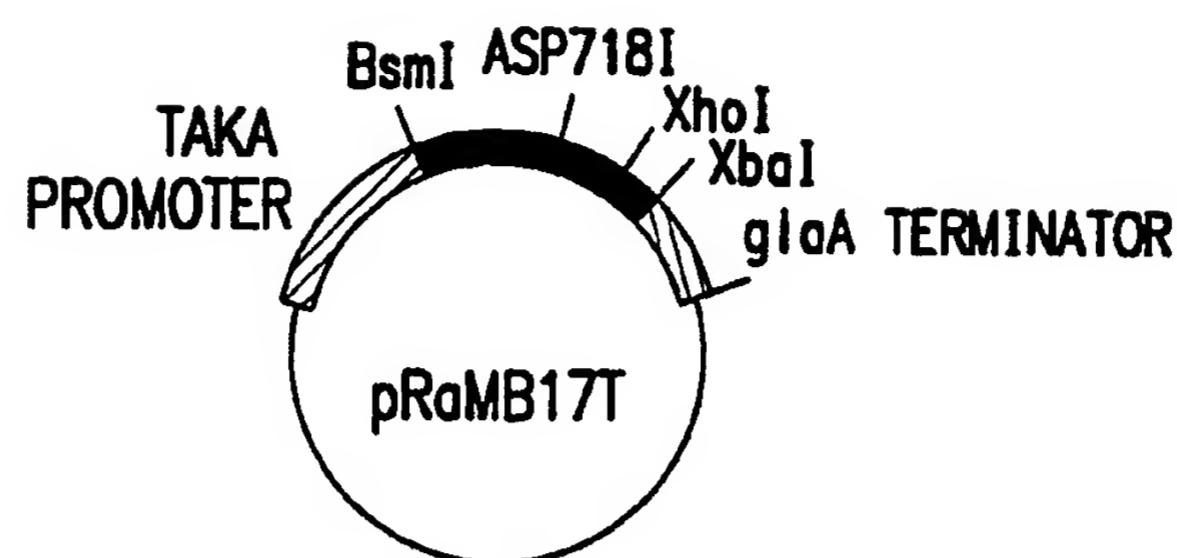
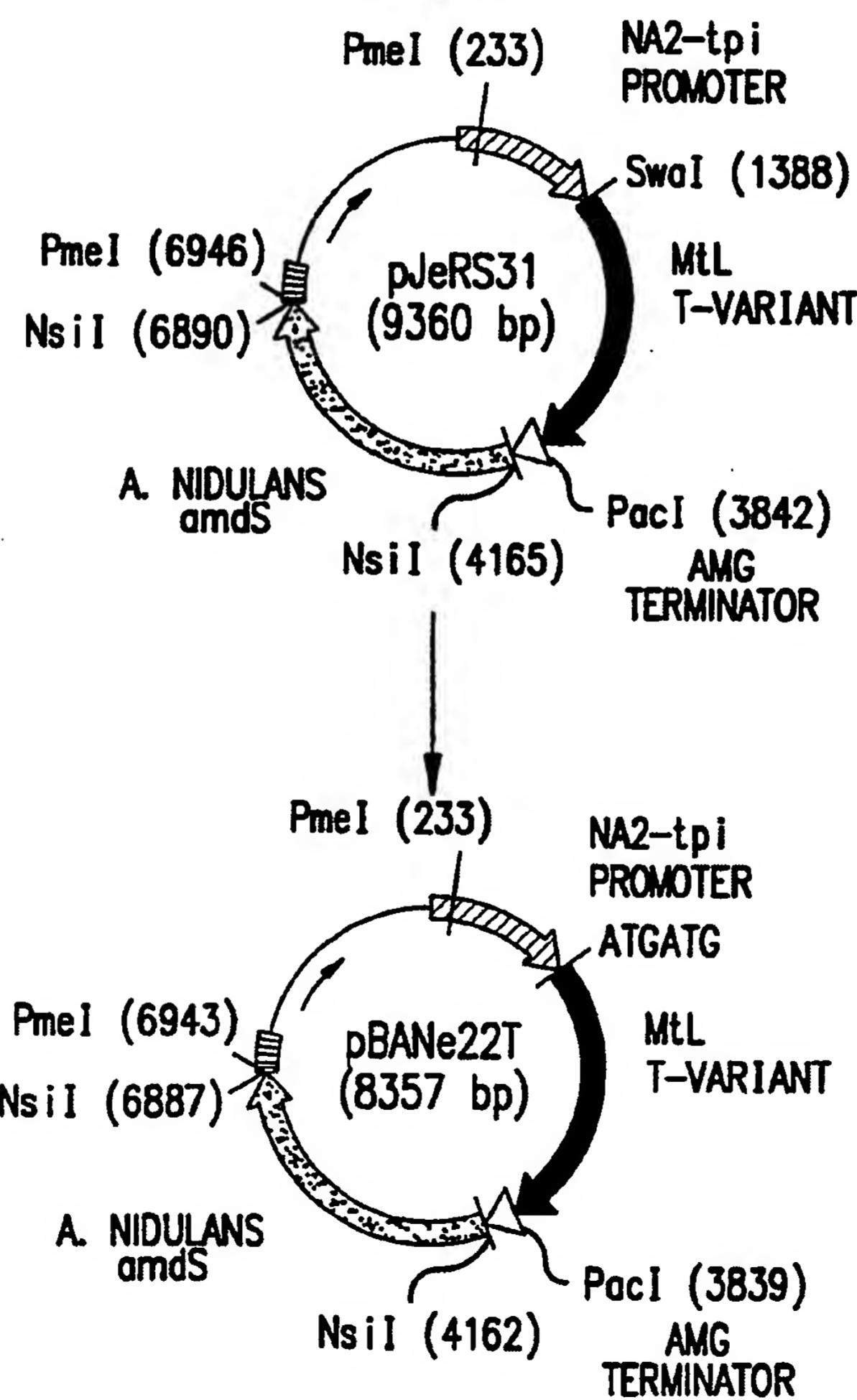


FIG.4



6/15

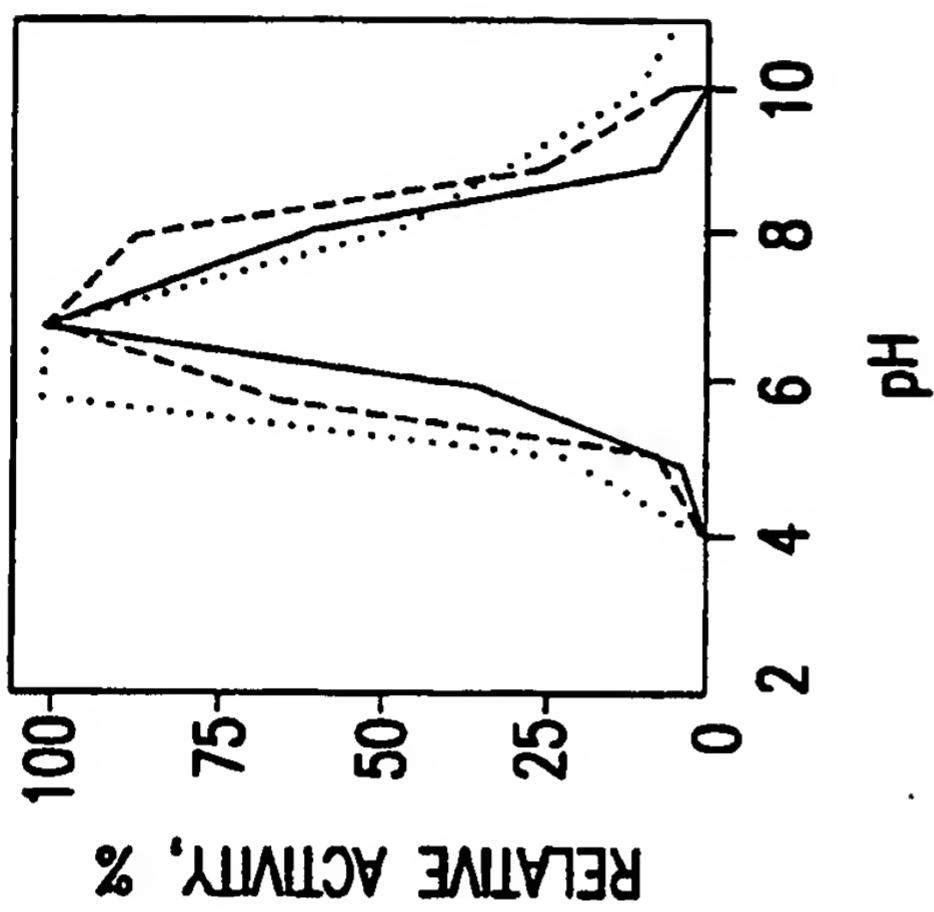


FIG. 5B

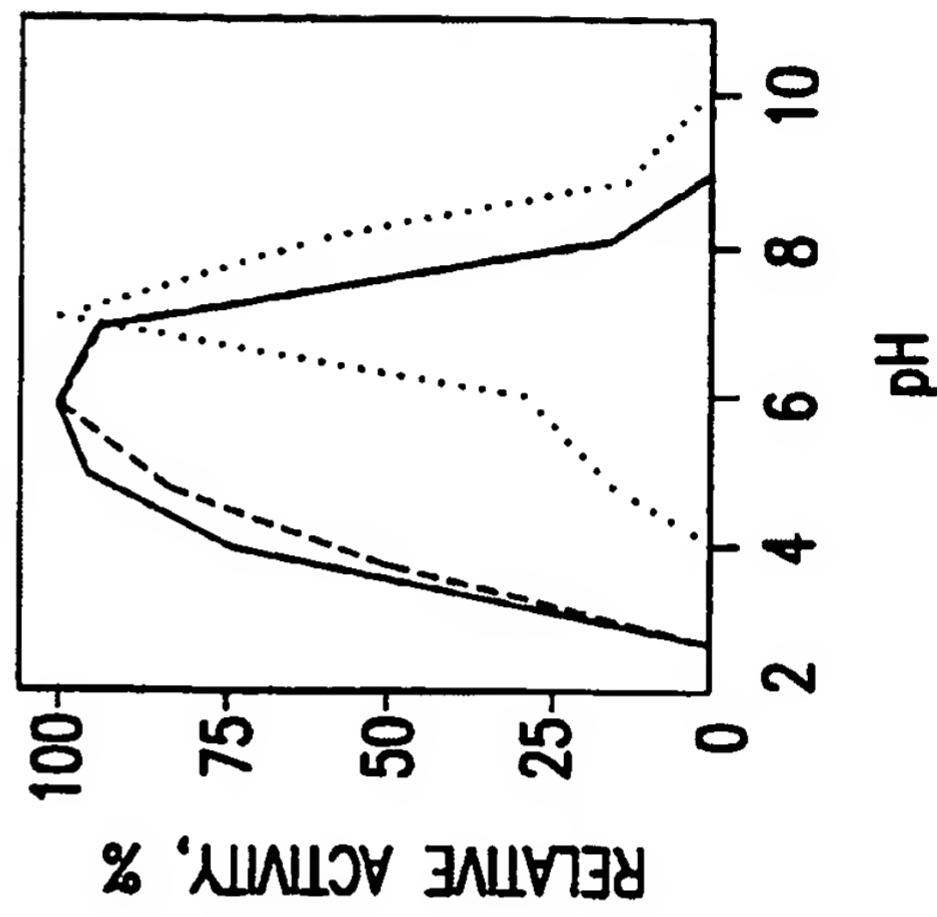


FIG. 5D

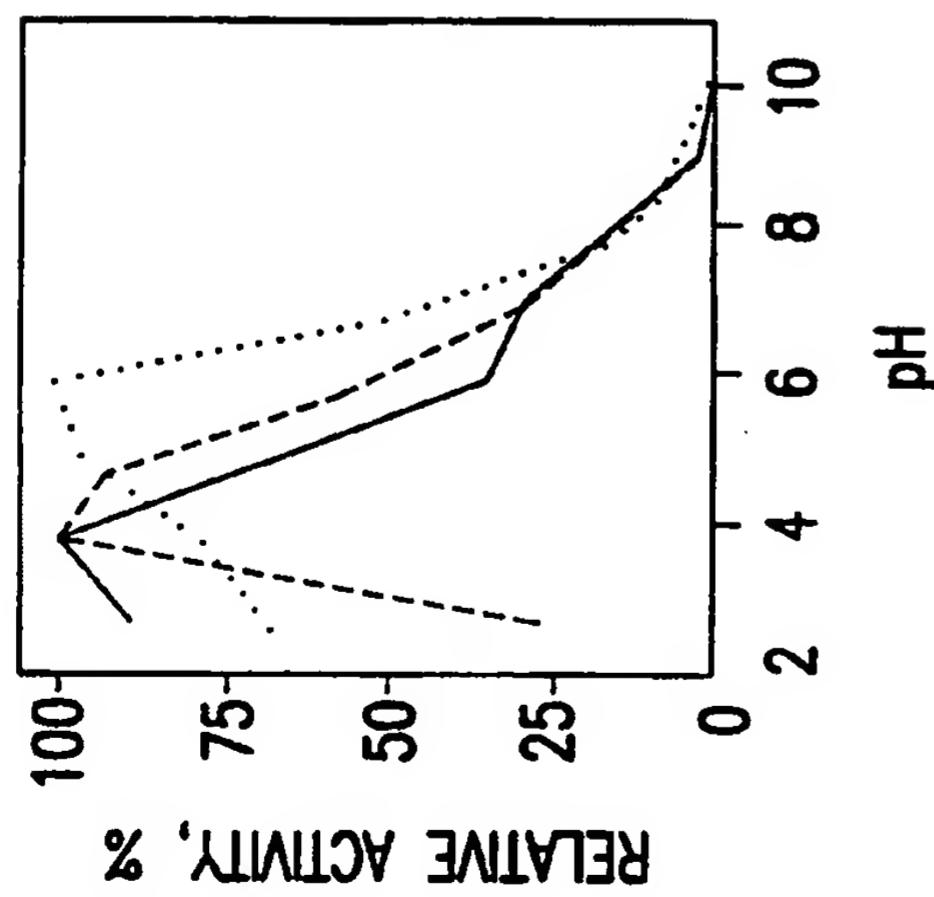


FIG. 5A

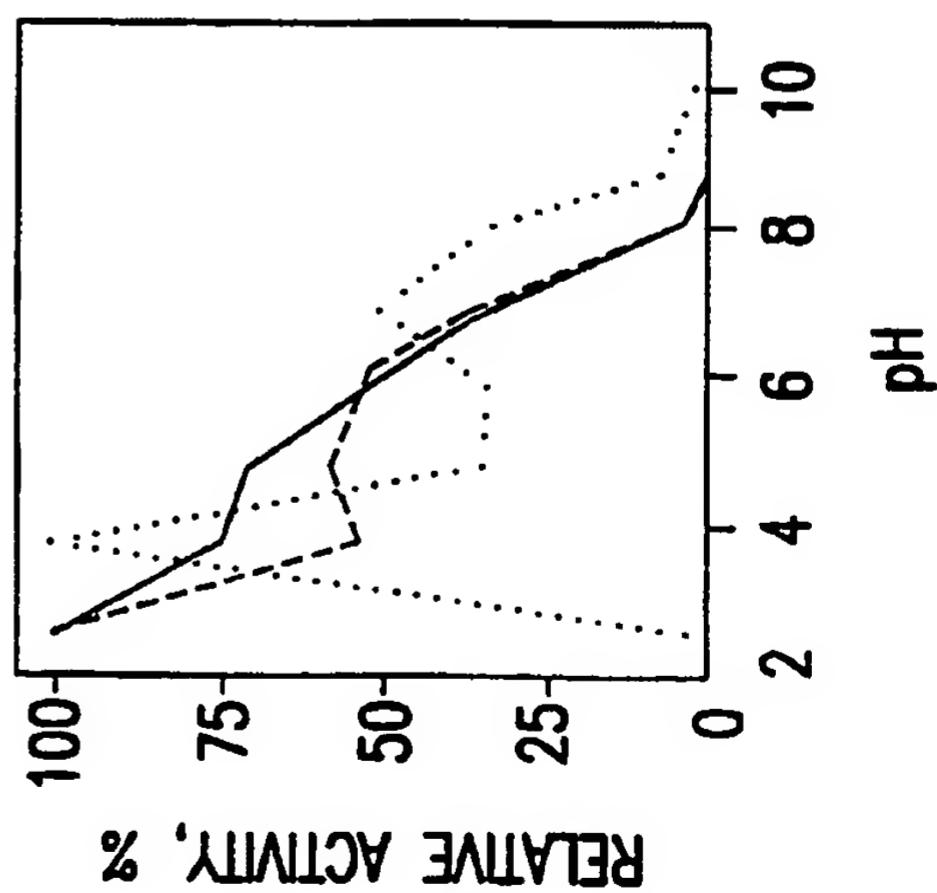


FIG. 5C

7/15

5' ATG CTT TCT AGC ATT ACC CTC CTA CCT TTG CTC GCT GCG GTC TCA ACC CCC GCC  
 ----- M L S S I T L L P L L A A V S T P A  
 87 96 105 114 123 132  
 TTT GCT GCC GTC CGC AAC TAT AAG TTC GAC ATC AAG AAC GTC AAT GTC GCT CCC  
 ----- F A A V R N Y K F D I K N V N V A P  
 141 150 159 168 177 186  
 GAT GGC TTT CAG CGC TCT ATC GTC TCC GTC AAC GGT TTA GTT CCT GGC ACG TTG  
 ----- D G F Q R S I V S V N G L V P G T L  
 195 204 213 222 231 240  
 ATC ACG GCC AAC AAG GGT GAC ACC TTG CGC ATT AAT GTC ACG AAT CAA CTC ACG  
 ----- I T A N K G D T L R I N V T N Q L T  
 249 258 267 276 285 294  
 GAC CCT AGT ATG CGT CGT GCC ACA ACG ATT CAT TGG CAT GGA TTG TTC CAA GCT  
 ----- D P S M R R A T T I H W H G L F Q A  
 303 312 321 330 339 348  
 ACT ACC GCC GAC GAG GAT GGC CCC GCA TTC GTC ACG CAA TGC CCT ATT GCG CAA  
 ----- T T A D E D G P A F V T Q C P I A Q  
 357 366 375 384 393 402  
 AAT TTG TCC TAT ACA TAC GAG ATC CCA TTG CGC GGC CAA ACA GGA ACC ATG TGG  
 ----- N L S Y T Y E I P L R G Q T G T M W  
 411 420 429 438 447 456  
 TAT CAC GCC CAT CTT GCG AGT CAA TAT GTC GAT GGA TTG CGA GGC CCT TTG GTC  
 ----- Y H A H L A S Q Y V D G L R G P L V  
 465 474 483 492 501 510  
 ATC TAT GAT CCA AAC GAC CCA CAC AAG TCG CGC TAC GAC GTG GAT GAT GCG AGC  
 ----- I Y D P N D P H K S R Y D V D D A S

FIG.6A

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8/15

573	582	591	600	609	618												
ACA	GTA	GTC	ATG	CTT	GAG	GAC	TGG	TAC	CAT	ACT	CCG	GCA	CCC	GTT	CTA	GAA	AAG
- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
T	V	V	M	L	E	D	W	Y	H	T	P	A	P	V	L	E	K
627	636	645	654	663	672												
CAA	ATG	TTC	TCG	ACT	AAT	AAC	ACC	GCT	CTG	CTC	TCT	CCT	GTT	CCG	GAC	TCG	GGT
- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
Q	M	F	S	T	N	N	T	A	L	L	S	P	V	P	D	S	G
681	690	699	708	717	726												
CTT	ATC	AAT	GGC	AAA	GGG	CGC	TAT	GTG	GGC	GGT	CCC	GCA	GTT	CCC	CGG	TCA	GTA
- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
L	I	N	G	K	G	R	Y	V	G	G	P	A	V	P	R	S	V
735	744	753	762	771	780												
ATC	AAC	GTA	AAA	CGT	GGG	AAA	CGA	TAT	CGC	TTG	CGC	GTA	ATC	AAC	GCT	TCT	GCT
- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
I	N	V	K	R	G	K	R	Y	R	L	R	V	I	N	A	S	A
789	798	807	816	825	834												
ATC	GGG	TCG	TTT	ACC	TTT	TCG	ATC	GAA	GGA	CAT	AGT	CTG	ACT	GTC	ATT	GAG	GCC
- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
I	G	S	F	T	F	S	I	E	G	H	S	L	T	V	I	E	A
843	852	861	870	879	888												
GAT	GGG	ATC	CTG	CAC	CAG	CCC	TTG	GCT	GTT	GAC	AGC	TTC	CAG	ATT	TAC	GCT	GGA
- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
D	G	I	L	H	Q	P	L	A	V	D	S	F	Q	I	Y	A	G
897	906	915	924	933	942												
CAA	CGC	TAC	TCT	GTC	ATC	GTT	GAA	GCC	AAC	CAA	ACC	GCC	GCC	AAC	TAC	TGG	ATT
- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
Q	R	Y	S	V	I	V	E	A	N	Q	T	A	A	N	Y	W	I
951	960	969	978	987	996												
CGT	GCA	CCA	ATG	ACC	GTT	GCA	GGA	GCC	GGA	ACC	AAT	GCA	AAC	TTG	GAC	CCC	ACC
- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
R	A	P	M	T	V	A	G	A	G	T	N	A	N	L	D	P	T
1005	1014	1023	1032	1041	1050												
AAT	GTC	TTT	GCC	GTA	TTG	CAC	TAC	GAG	GGA	GCG	CCC	AAC	GCC	GAA	CCC	ACG	ACG
- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
N	V	F	A	V	L	H	Y	E	G	A	P	N	A	E	P	T	T

FIG.6B

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9/15

1059	1068	1077	1086	1095	1104												
GAA	CAA	GGC	AGT	GCT	ATC	GGT	ACT	GCA	GTG	GTT	GAA	GAG	AAC	GTG	CAT	GCG	CTC
- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	
E	Q	G	S	A	I	G	T	A	L	V	E	E	N	L	H	A	L
1113	1122	1131	1140	1149	1158												
ATC	AAC	CCT	GGC	GCT	CCG	GGC	GGC	TCC	GCT	CCC	GCA	GAC	GTG	TCC	CTC	AAT	CTT
- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	
I	N	P	G	A	P	G	G	S	A	P	A	D	V	S	L	N	L
1167	1176	1185	1194	1203	1212												
GCA	ATT	GGG	CGC	AGC	ACA	GTT	GAT	GGG	ATT	CTT	AGG	TTC	ACA	TTT	AAT	AAC	ATC
- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	
A	I	G	R	S	T	V	D	G	I	L	R	F	T	F	N	N	I
1221	1230	1239	1248	1257	1266												
ATC	TAC	GAG	GCT	CCT	TCG	TTG	CCC	ACG	CTC	TTG	AAG	ATT	TTG	GCA	AAC	AAT	GCG
- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -		
K	Y	E	A	P	S	L	P	T	L	L	K	I	L	A	N	N	A
1275	1284	1293	1302	1311	1320												
AGC	AAT	GAC	GCC	GAT	TTC	ACG	CCA	AAT	GAG	CAC	ACT	ATC	GTA	TTG	CCA	CAC	AAT
- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -		
S	N	D	A	D	F	T	P	N	E	H	T	I	V	L	P	H	N
1329	1338	1347	1356	1365	1374												
AAA	GTT	ATC	GAG	CTC	AAT	ATC	ACC	GGA	GGT	GCA	GAC	CAC	CCT	ATC	CAT	CTC	CAC
- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -		
I	V	I	E	L	N	I	T	G	G	A	D	H	P	I	H	L	H
1383	1392	1401	1410	1419	1428												
GGC	CAT	GTG	TTT	GAT	ATC	GTC	AAA	TCA	CTC	GGT	GGT	ACC	CCG	AAC	TAT	GTC	AAC
- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -		
G	H	V	F	D	I	V	K	S	L	G	G	T	P	N	Y	V	N
1437	1446	1455	1464	1473	1482												
CCG	CCA	CGC	AGG	GAC	GTA	GTT	CGT	GTC	GGA	GGC	ACC	GGT	GTG	GTA	CTC	CGA	TTC
- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -		
P	P	R	R	D	V	V	R	V	G	G	T	G	V	V	L	R	F
1491	1500	1509	1518	1527	1536												
AAG	ACC	GAT	AAC	CCA	GGC	CCA	TGG	TTT	GTT	CAC	TGC	CAC	ATT	GAC	TGG	CAC	TTG
- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -		
K	T	D	N	P	G	P	W	F	V	H	C	H	I	D	W	H	L

**FIG.6C**  
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10/15

1545        1554        1563        1572        1581        1590  
GAG GCT GGG CTC GCA CTT GTC TTT GCC GAG GCC CCC AGC CAG ATT CGC CAG GGT  
-----  
E A G L A L V F A E A P S Q I R Q G

1599        1608        1617        1626        1635        1644  
GTC CAG TCG CTG CAG CCC AAC AAT GCC TGG AAC CAG CTC TGC CCC AAG TAC GCG  
-----  
V Q S V Q P N N A W N Q L C P K Y A

1653        1662  
GCT CTT CCT CCC GAT TTG CAG T 3'  
-----  
A L P P D L Q

## FIG.6D

11/15

gctagtttggtcaccgtcggtttccaccccttcaaccccccgttagtgcggtaaggcataccctca  
80  
 atctggtgtgtgaggcacgtccctccaggcagatgtacatcgaggcatgtcgccatcggcgttatcc  
160  
 gatgcgcgcacatccatgggtttcccggttccggggatatttcggatgtggaaaggagaaaagg  
240  
 cggaccggctccaccccggttccggggatgtcgacttccaaacatcgagaacaggaaagggtgtcgcc  
320  
 agggggggggggaaacatgttgatacggactgcggcccttcgtcgacttgtctcaggttctctcgac  
400  
 cgtaatgtcttataaaaacgggacttcgtcgacttgtctcaggttctctcgacccaggccaggctt  
480  
 gcctgagccacctgaggccacctcatcttcaggatcaagtcaactcatcgatgtgtctatcgag  
560  
 tcggctcccccccttaccacaaacATGAAGTCCTCATCAGGCCGGACGCTTTGGTGGCATTCTCAC  
MetLysSerPheIleSerAlaAlaThrLeuValGlyIleLeuThrProSer  
-30  
 TTGCTGCTGCCCTCCATCCACCGCTGAGCAGGGAGGGCAGGCCGTGAA  
ValAlaAlaAlaProSerThrProGluGlnArgAspLeuValProIleThrGluArgGluAlaValLy  
-3  
 GGCTCGCCAGCAGCTGCAACACCCCCAGCAACCGGGCGTGGACTGACGGATAACGACATCAACAC  
SA1aArgGlnGlnSerCysAsnThrProSerAsnArgAlaCysTrpThrAspGlyTyrAspIleAsnThr  
AspTyrGluV  
25  
 TGGACAGCCCCGACACGGGTGTTGGCCGgttagtgcgttttttttttttttttttttttttttttt  
a1AspSerProAspThrGlyValValArgPro  
35

**FIG. 7A**

12/15

aatactgcaaccctaaggcaggactgacatgcacagTACACTCTGACTCTCACCGAAGTCGACAACTGGACCGAACCTG TyrThrLeuThrGluValAspAsnTrpThrGlyProA	960 50
ATGCCGTCAAGGAGGTCATGCTGGTTAACAGtacggccaccccttttgtccttaaggatctgggtgatgtgcgt spGlyValLysGluLysValMetLeuValAsnA	1040 62
cgttgcctcgagagactgaccgcggcttgctgcaggATAGTATAATCGgtattaaattataccgcctgcctccag snSerIleIleG	1120 66
cagccccagcagctcgagaaagggtatctgaagttagtcaggcctgctgaccctgaccggccaaacccaccatagGACCAA 1yProt	1200 68
CAATCTTGCGGACTGGGGCACACCGATCCAGGTAAACGATCATTAAACAACCTCGAGACCACGGgttatgtctgctgtcttg hrIlePheAlaAspTrpGlyAspThrIleGlnValThrValIleAsnAsnLeuGluThrAsnG1	1280 89
ctcttgcctctcgccgactaataataactcaactcggtggaaaaacaggCACGTCGATCCACTGGCACGGACT yThrSerIleHisTrpHisGlyLe	1360 97
GCCCCAGAACCAACCTGCACGACGGGCCAACGGTATCACCGAGTGCCGATCCCCCAAGGGAGGGAGGAAGG uHisG1nLysGlyThrAsnLeuHisAspGlyAlaAsnGlyIleThrGlyCysProIleProProLysGlyArgLysV	1440 124
TGTACCGGTTCAAGGCTCAGCAGTACGGGACGGCTGGTACCACTCGCACTTCTGGCCAGTACGGCAACGGCTGGTC a1TyrArgPheLysAlaG1nGlyTyrGlyThrSerTrpTyrHisSerHisPheSerAlaGlnTyrGlyAsnGlyValVal	1520 150
GGGCCATTCAACGGACCGGGCTCGCTGCCGTACGACACCGACCTGGGTGTGTTCCCCCATCAGCGACTACTA GlyAlaIleGlnIleAsnGlyProAlaSerLeuProTyrAspThrAspLeuGlyValPheProIleSerAspTyrTyrTy	1600 177

**FIG. 7B**

CAGCTCGGCCGACGAGCTGGAACTCACCAAGAACCTGGGGCCCTTCAGCGACAACGTCCCTGTTCAACGGCACGG  
rSerSerAlaAspGluLeuValGluleuThrLysAsnSerGlyAlaProPheSerAspAsnValLeuPheAsnGlyThrA 1680  
204

CCAAGCACCCGGAGACGGGGCAGACTACGCCAACCGTGACGCTCACCCCCGGGGCACCCGCCTGGCCTGATC 1760  
1aLysHi sProGluThrGlyGluGlyGluTyrAlaAsnValThrLeuThrProGlyArgArgHi sArgLeuArgLeuIle 230

AACACGTCGGTCGAGAACCACTCCAGGTCTCGCTCAACCACCCATGTCATCATGCCGCCGACATGGTGCCTGGCGT 1840  
AsnThrSerValGluAsnHi sPheGlnValSerLeuValAsnHisThrMetCysIleIleAlaAlaAspMetValProVa 257

CAACGCCATGACGGGTGACAGGCCCTCTCGGCCAGCGTTACCGATGTCGTATCGAACGCCAACCGAACGCC 1920  
1AsnAlaMetThrValAspSerLeuPheLeuGlyValGlyValArgTyrAspValValValAsnArgThrProG 284

GGAACTACTGGTTAACGGTCACATTGGGGCCCTGCTCTGGGGCCAGGAATCCCTACCCGGGCCATCTTC 2000  
1yAsnTyrTrpPheAsnValThrPheGlyAlaProGlyGlyLeuLeuCysGlyGlySerArgAsnProTyrProAlaAlaIlePhe 310

CACTACGGCCCCGGCCGCCACGGACGGGGCAAGGGCCCTGCTGGGGCCGGTCGACCAACTGCCCTGGAACCTCCCAA 2080  
Hi sTyrAlaGlyAlaProGlyGlyProProThrAspGlyGlyLysAlaProValAspHi sAsnCysLeuAspLeuProAs 337

CCTCAAGCCCCGTGTCGTGGCCCGACGTGCCCTGAGGGCTCCAGGGGGCCGACAACACGGCTCACCTCG 2160  
nLeuLysProValAlaArgAspValProLeuSerGlyPheAlaLysArgAlaAspAsnThrLeuAspValThrLeuA 364

ACACCAAGGGCACGGCCCTGTTCTGAAAGGTCAACGGCAGGCCATCAACATCGACTGGGGAGGGCCGTCGTGGAC 2240  
spThrThrGlyThrProLeuPheValTrpLysValAsnGlySerAlaAlaAsnValAspTrpGlyArgAlaValValAsp 390

TACGTCCCTCACGGCAGAACACAGCTCCACCCGGGTACACATTGTCGAGGTGAACGGAGCTGATCAAGgtaaaaaaag 2320  
TyrValLeuThrGlnAsnThrSerPheProProGlyTyrAsnIleValGluValAsnGlyAlaAspGln 413

13/15

**FIG. 7C**

ggaccgcagggtgctgcaagtacacccctcgccctccctgttactaccctccaaacctcccc	2400	
ctaattcacttaaaaggccgatcaagactqaccgcggcttccttaataactaccctccaaacctcccc	2480	
TrpSerTyrTripleuIleGluAsn	421	
GATCCGGCACCTTCACCCCATCCGATGCCACCTGCACgtaaagttggatacatatatacatat	2560	
AspProGlyAlaProPheThrLeuProHisProMetHisLeuHis	436	
tgcttgcgtcgctcccttaaaaataaaaaataaaaaaaagGCCACGACTTTACGTGCTGG	2640	
GlyHiAspPheTyrValLeuG	444	
GCCGCTCGCCCCGACGAGTCGGCATTGCTGGATCCGGCGACGGCGCTGCTGAGCGGG	2720	
1yArgSerProAspGluSerProAlaSerAsnGluArgHisValPheAspProAlaArgAspAlaGlyLeuSerGly	470	
GCCAACCCTGTGGGGGACGGTGTGGATGCCGGCTTCGGGTGGTGCTGCTGCCCTCCGGACAACCCGGG	2800	
AlaAsnProValArgArgAspValSerMetLeuProAlaPheGlyTrpValValSerPheArgAlaAspAsnProGly	497	
GGCCTGGCTCACTGCCACATGGCACGGTCTCGGCCCTGGCGACTCGAGGCCGACCGACC	2880	
yAlaTrpLeuPheHisIleAlaTrpHisValSerGlyLeuGlyValValTyrLeuGlyValAspAspAspAlaAspArgLeuCysAlaAspAspLeuAspAspAlaAspAspSerGlyLeuLysHisArgTrpValArgArgTyrTrpProThrAsn	524	
TGGGGGGCGTCTGGACGGACGCCCTCGACCGACGCGCTACTGGCCTACCGGACTCGGCGGCTAC	2960	
euArgGlyAlaValSerAspAlaAspAspSerGlyLeuLysHisArgTrpValArgArgTyrTrpProThrAsn	550	
CCCTACCCCAAGTCCGACTCGGGCCTCAAACACCGCTGGGTCAAGGGCGAGTGGCTGGAGGAGGG	3040	
ProTyrProLysSerAspSerGlyLeuLysHisArgTrpValGluGlyGluTrpLeuValLysAla***	573	

14/15

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EIG. 7 D

15/15

ggaaaaaggaaaccaaagaggggggggctagttccatttttgcttttttgttgccttgcgtggcg 3120  
ttccctgtaaggagaaggggccccaaatcgcagtggtgtgatcggtaaatatcaagagatct 3192

**FIG. 7E**

# INTERNATIONAL SEARCH REPORT

Internal Application No  
PCT/US 96/14087

**A. CLASSIFICATION OF SUBJECT MATTER**  
IPC 6 C12N15/53 C12N9/02

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 263, no. 2, 15 January 1988, MD US, pages 885-896, XP002023112 U.A.GERMANN ET AL.: "Characteristics of two allelic forms of Neurospora crassa laccase" see the whole article, especially figure 4. --- X JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 265, no. 25, 5 September 1990, MD US, pages 15224-15230, XP002023164 Y.KOJIMANI ET AL.: "Cloning, sequence analysis, and expression of ligninolytic phenoloxidase genes of the white-rot basidiomycete Coriolus hirstus" see figure 3 --- -/-	1-9,15, 16, 21-25, 33-38
X		1-9,15, 16, 21-25, 33-38

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

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\*&\* document member of the same patent family

Date of the actual completion of the international search

Date of mailing of the international search report

20 January 1997

31.01.97

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Authorized officer

Cupido, M

## INTERNATIONAL SEARCH REPORT

Internat'l Application No  
PCT/US 96/14087

C(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
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X	WO,A,92 01046 (VALTION TEKNILLINEN TUTMIKUSKEKKESKUS) 23 January 1992  see the whole document	1-9,15, 16, 21-25, 33-38
P,X	WO,A,95 33836 (NOVO NORDISK BIOTECH, INC.) 14 December 1995  see the whole document, especially SEQ ID NOs 1 and 2 ---	1-9,15, 19-25, 33-38
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A	CELL, vol. 76, no. 2, 28 January 1994, NA US, pages 403-410, XP002023113 C.ASKWITH ET AL.: "The FET3 gene of S.cerevisiae encodes a multicopper oxidase required for ferrous iron uptake" see figure 5 -----	1-9

**INTERNATIONAL SEARCH REPORT**

Internat'l Application No

PCT/US 96/14087

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		CN-A-	1133067	09-10-96
		EP-A-	0719337	03-07-96
		FI-A-	961250	18-03-96
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WO-A-9201046	23-01-92	NONE		-----
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		JP-A-	5268950	19-10-93
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